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EDITOR-IN-CHIEF
C.-E. A. Winslow

It is characteristic of Science and Progress that they continually open new fields to our vision.—Pasteur.

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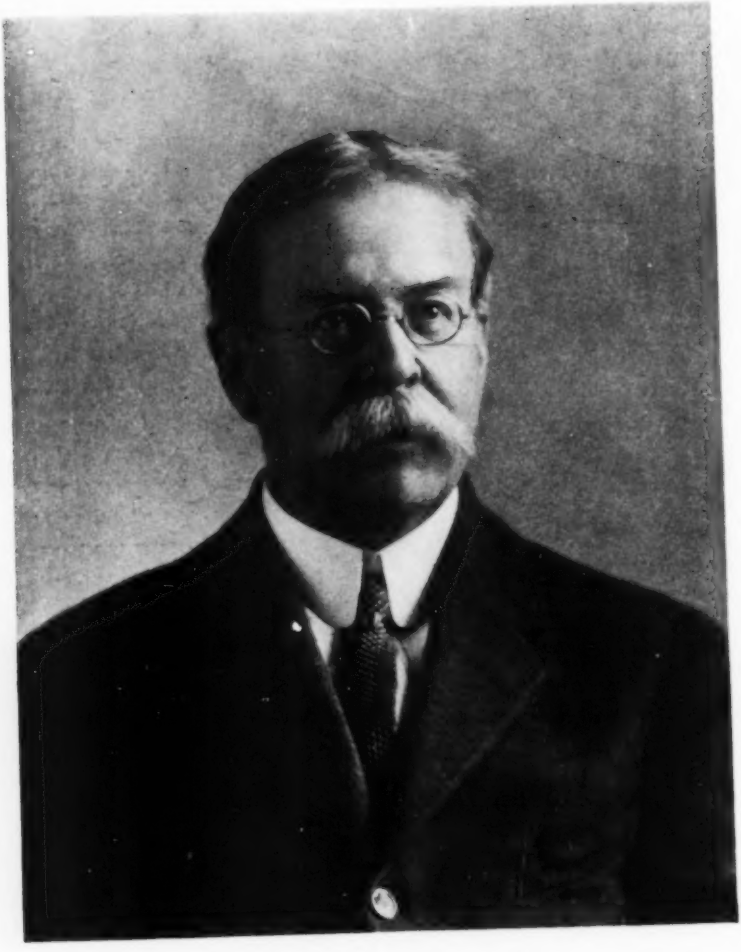

William Thompson Sedgwick

WILLIAM THOMPSON SEDGWICK

1855–1921

William Thompson Sedgwick, the father of the modern public health movement in America, was born at West Hartford, Connecticut, December 29, 1855. He graduated from the Sheffield Scientific School of Yale University in 1877, his first contribution to scientific literature being a study of the local flora, in collaboration with his college chum and life-long friend, E. B. Wilson. He began the study of medicine, but, dissatisfied with the haphazard medical education of the time, discontinued his course a short time before he would have received his degree. He taught physiological chemistry under Chittenden at the Sheffield Scientific School in 1878–1879, and in 1879 accepted a fellowship in biology at Johns Hopkins where he came under the influence of Martin, to receive from him the vision of biology as a broad and liberal science, a vision which Martin brought over from the England of Huxley and transmitted through Sedgwick and Sedgwick's pupils to thousands of students in this country.

Sedgwick was made assistant in biology at Baltimore and received the degree of Ph.D. in 1881. In the winter following the reception of his doctorate and on the anniversary of his birth, December 29, 1881, he was married to Mary Catherine Rice of New Haven, the beginning of thirty-nine years of a relationship as complete and as beautiful as ever existed between man and wife. Mrs. Sedgwick not only gave to her husband a rare personal devotion which made his health and his comfort and the success of his career a constantly controlling motive, but her artistic tastes and rich temperament kept a warmth and color in his life which made it impossible for Sedgwick ever to feel those limitations which sometimes accompany a life of intellectual concentration, limitations which Charles Darwin, for example, felt so pathetically in his later years.

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Sedgwick found his career in 1879, his wife in 1881, and the institution to which he and his wife devoted their lives with a rare ardor in 1883. Francis Walker, who was at this time beginning his brilliant service as president of the Massachusetts Institute of Technology, had known Sedgwick as a student at Yale, and with a characteristically broad view of technological education, called him to the Institute in 1883 as Assistant Professor of Biology. He became Associate Professor in 1884, and Professor in 1891 and was head of the department (later known as the Department of Biology and Public Health) until his death.

In the present prosperous state of scientific education, it is a little difficult to realize what the Institute of Technology meant to its protagonists. In those early days of doubt and difficulty the Institute became a symbol, an Ark of scientific education to Walker and the little band who fought for it at his side. Sedgwick was one of Walker's closest friends and, like Walker and so many of his faculty, was inspired by a devotion to the ideals of the Institute which is bestowed upon church and nation more often than upon an educational institution.

Sedgwick's original bent was toward physiology and his first important scientific contribution at the Institute was a study of the dangers of gas poisoning, conducted in collaboration with William Ripley Nichols. These were the golden days of the birth of bacteriology, however, and when Nichols died while on a visit to the European universities some tubes of Koch's strange new gelatin medium were brought back to the Institute with his personal effects. Sedgwick was quick to realize the possibilities of the new science and from that time on his own investigations and the energies of his department were focused more and more on bacteriology. The medical applications of the subject were being developed by Welch at Baltimore and by Prudden and Biggs and Park in New York, but Sedgwick's training and natural aptitudes made him the pioneer in the broader biological aspects of the new subject. When the Massachusetts State Board of Health was reorganized and the Lawrence Experiment Station was established in 1888, he was appointed biologist to the Board and with Mills, Drown and

Mrs. Richards and their pupils, Hazen, Whipple, Fuller and Jordan, he laid the foundation of modern sanitary science in its bacteriological and engineering aspects, as distinct from those which deal with the problems of the pathology and diagnosis of disease. His contributions to epidemiology in the study of water and milk-borne epidemics, conducted at this time, were of the highest scientific importance. The growth of the whole public health movement in America was, from 1890, connected in an intimate fashion with the development of the Department of Biology and Public Health at the Institute and of the School for Health Officers conducted in coöperation with Harvard University during recent years. It would be difficult to name any important health activity, investigative, administrative or educational, to which Sedgwick's pupils have not contributed in an important degree. It may be fairly said that he created the new field of non-medical sanitary science. Public health began as a branch of medicine but Sedgwick has taught America, and, through his pupils, is now teaching Europe that the two fields are intersecting but distinct, and that sanitary engineers, bacteriologists and even health administrators may be trained for the highest type of public service without passing through the established course which leads to the medical degree. The last important idea, which he put forward only a few months before his death, was the suggestion of a bifurcated course, based on the same two years of pre-clinical work, but leading in the last two years to the alternative degrees of Doctor of Medicine or Doctor of Public Health; and this suggestion was the logical development of his life work.

Aside from a multitude of important technical papers and addresses, Sedgwick was the author, or joint author, of five books which admirably express the more important interests of his professional life. "General Biology," published with E. B. Wilson in 1886 crystallized in effective form the viewpoint derived, through Martin, from Huxley of biology as a broad and fundamental discipline dealing with the underlying phenomena of protoplasmic action; and no single work has perhaps had so large an influence upon the teaching of the biological sciences

in the United States. The "Life and Letters of William Barton Rogers" (1896), in the preparation of which Sedgwick assisted President Roger's widow, was a labor of love which expressed all the loyalty of the Technology faculty and alumni to the great founder of the Institute. "Principles of Sanitary Science and the Public Health" (1902) was Sedgwick's most important literary production, a book which is still the best existing epitome of the principles of sanitary science and which many academic generations have found "as interesting as a novel." "The Human Mechanism," a textbook for schools and colleges, published with Theodore Hough in 1906, marked Sedgwick's return to his earlier interest in physiology and personal hygiene; and "A Short History of Science," published with H. W. Tyler in 1917, placed in permanent form the broad historical sense and the keen love of origins which were always among the greatest charms of Sedgwick's courses.

Sedgwick's scientific attainments received recognition in the conferring of the honorary degrees of Sc.D. by Yale in 1909, and LL.D. by the University of Cincinnati in 1920, as well as in election to the American Academy of Arts and Sciences and the American Philosophical Society. He was appointed a member of the Advisory Board of the United States Hygienic Laboratory in 1902, and later received a commission as Assistant Surgeon General in the United States Public Health Service. He was a member of the International Health Board of the Rockefeller Foundation. He was a founder and first president of the Society of American Bacteriologists and our organization owes its establishment and its broad charter more perhaps to him than to any other individual. He served also as president of the American Society of Naturalists, the American Public Health Association, and the New England Water Works Association.

Sedgwick's interests were, however, never narrowly bounded by his own technical field. Wherever educational or civic problems were to be solved he was ready to serve. A score of progressive movements in Massachusetts numbered him among their leaders. He was president of the board of trustees of Sharon Sanatorium from 1902 and a member of the Public Health

Council of Massachusetts from its inception. He was a trustee of Simmons College from its foundation in 1899. He was chairman of the Pauper Institutions' Trustees of the city of Boston in 1897–1899. He was a leading figure in the fight for Civil Service Reform, president of the Boston Civil Service Reform Association in 1900, and of the State Association in 1901. Finally, as curator of the Lowell Institute since 1897 he became perhaps more widely known to the citizens of Boston than in any other capacity. He did not confine himself to the abstract task of securing for Boston contracts with the most brilliant teachers of American and European thought; he was almost nightly on hand to act as a personal host and to give the problems of heating and lighting and ventilation an individual attention which made Huntington Hall famous throughout the country.

In all these works of public service Sedgwick was unwearied, until the very day and hour of his death (January 25, 1921). On Saturday he gave a dinner to some thirty of his colleagues and pupils in honor of a former student who was going abroad on a public health mission, and never was he more at his best in wisdom and courage and enthusiasm. On Monday he was at his office as usual; the writer will always cherish as one of his most precious possessions a long letter written on this day, about a projected journey, full of the sound counsel and the detailed practical advice which "The Chief" always found time to give to his old students. On Tuesday evening he attended a meeting in the interest of a plan for the formation of a state university, walked home enjoying the keen, frosty air of the Boston winter and on his arrival, after a word of cheer to Mrs. Sedgwick, succumbed in a moment to an attack of an affection of the heart which had for years threatened but never shadowed his life. He died without regaining consciousness, a "Happy Warrior" in the fight against ignorance and suffering and disease.

Sedgwick was a pioneer in American science and a zealous public servant; but it was as a teacher that he stood supreme. On the lecture platform, as in the intimacy of his laboratory, he had the gift, as rare as it is beneficent, of seizing the imagination,

kindling the enthusiasm, inspiring the will. He was no orator, but he compelled by the force of a ripe intellect, a genial philosophy and an unswerving ideal. He had the instinct for the vital point; and in the midst of all his busy life he never failed to gauge the strength and the weakness of each individual student. He was pitiless to the specious and the slipshod, and if his students did not learn to think honestly and clearly they had only themselves to blame.

Sedgwick's most notable intellectual quality was breadth of vision. He saw every fact in relation to a hundred other phenomena and he was at his very best with a small group of students, following out in the experimental vein a line of thought which might lead from the structure of plant tissue to the domestic life of ancient Rome, and then to some fundamental problem in philosophy or ethics. The Bible, the Greek classics and the poets and essayists of England were always fresh in his mind to furnish an allusion. He and Mrs. Sedgwick had travelled in Europe, widely and in unusual by-paths; and he travelled with eyes so wide open and interest so keen that he saw more and enjoyed more in a month than many a self-centered tourist can compass in a year. (One of the things his friends love best to remember is the satisfaction he derived from his trip to Europe last summer as exchange professor at Leeds and Cambridge.) The whole world, past and present, was in the background of his thoughts. He would take a simple fact and turn it this way and that, and play with it, and toss it in the air, so that it caught the light from a hundred different sources. No one who has ever heard him discuss with a class by the Socratic method the question, "What is truth, and why do we value it so highly?" can ever forget that lesson in clear and straightforward and constructive reasoning. The Institute is a busy place and no man on its faculty was more active than Sedgwick in multifarious lines of public service, yet he was always calm, serene and unhurried. If it could ever be said of any man, it was true of him that he saw life steadily and saw it whole.

Sedgwick had knowledge and wisdom, but, when all is said and done, it is moral qualities which mark the great teacher.

"Faith, Hope and Charity" are the things that count in the long run; and these virtues were his in bountiful degree.

He had an abiding faith in the general scheme of things, a faith based firmly on the biologists' knowledge of the great underlying forces which have brought us up from the slime of the rockpools and which will yet carry us to heights undreamed of. He "Accepted the Universe," he trusted "that power not ourselves that makes for righteousness." His courage was absolute and instinctive. When he saw the truth he followed it. In times of doubt and and hesitation, one turned to him as to a well of clear water in the wilderness.

His optimism was no less notable a characteristic. He believed in his students and gave them responsibilities that seemed far beyond their powers, but almost always they "made good." Scores of young men who bore every sign of mediocrity were re-made and launched on successful careers by the sheer power of his confidence. In his public life Sedgwick saw much of the seamy side of American politics, yet he would approach a case-hardened politician with the assumption that they shared the same high ideals of social responsibility, and here too his optimism often bore surprising fruit.

Finally, Sedgwick loved not only mankind but he loved his fellowmen, which is a rarer and more precious gift. He established human relations with extraordinary facility. He knew his choreman and his elevator boy and the janitors at the Institute as human beings. One of the most characteristic things he ever did was the giving of a dinner, when his summer home at Seal Harbor was completed, to all the carpenters and masons, his friends and fellow townsmen of the Maine village who had labored honestly to build it. Above all, it was to his students that he gave of this power of warm personal sympathy and comprehension. One thinks always of "Rugby Chapel" as the ultimate tribute to a great teacher. About Sedgwick, however, there was something so much closer and more intimate that the quotation dies on one's lips. The master of Rugby was far off on the snowy heights. Sedgwick was in the midst of the rush of life and he held us by the hand. Arnold thought of his father

as a teacher. We who were Sedgwick's "boys" will think of our Chief as of a second father.

Yet he led us to the heights no less surely, if he led us always in warm and human fashion. It was not necessary for him, like the eastern sages, to go into the wilderness to learn the secret of selflessness. He knew it always. After a long and intimate talk with a student, he ended with the words "I think you can be a very useful man." Not a rich man, not a successful man, not an influential man; a useful man. That was his secret. I believe that never in his life, in matters great or small, did he say to himself, "Is it pleasant to do this?" "Is it to my interest to do this?" but only "Will this be useful?"

So, in this time, when the world seems very barren without his personal presence, his pupils and his colleagues and his friends can have but one thought—to labor more diligently and untiringly, that Sedgwick's spirit of service through knowledge may still bear fruit throughout the coming years.

C.-E. A. WINSLOW.

THE MAIN LINES OF THE NATURAL BACTERIAL SYSTEM

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I am happy to see how very intelligently and thoroughly my proposition for a natural bacterial system has been discussed by the Committee appointed by the Society of American Bacteriologists (1917). This warrants the hope that some day, when the single groups of bacteria have been sufficiently studied, the bacteriologists of the different countries may fortunately come to an agreement about a fully satisfactory bacterial classification. On the other hand, the Committee does not let me hope that we might agree also upon a more practical system of nomenclature than that employed at present in bacteriology, and I therefore feel impelled to object against the rather severe criticism that the Committee has passed on my efforts in this direction.

The basis of every science is, next to exact investigations, to throw the greatest possible clearness in the terms to be used. But science does not consist in pedantically following old-established rules. On the contrary, hardly any important progress is ever accomplished without disregarding some of them. Let us therefore, as we are now building up a new science, try to avoid the monstrous mistake committed by zoölogists and botanists in coining rather unmeaning terms which are apt to cause the greatest difficulties for the memory. Out of regard for posterity, who probably will find themselves confronted by thousands of bacterial species, we have to provide for a certain intrinsic logic in the nomenclature. No human being would now-a-days be able to recollect chemistry, were it not that in due time there had been prepared such an excellent nomenclature that the

name of a chemical compound can be derived directly from the formula. Even if the principles of chemical nomenclature cannot be applied to bacteriology, there is no reason here to form the names servilely upon the principles of Linnæus, and it is so much the more meaningless to do so as the Committee has already in its classification of bacteria discarded these principles on the most important point, in giving the biological qualities precedence over the morphological.

In bacteriology as soon as the purely morphological principle of classification is abandoned, the relatively few purely morphological generic names do not suffice, but we must necessarily form a whole series of new generic names. Precisely in this connection I think I have displayed a great deal of conservatism by simply adding to the old designations a prefix which characterizes the genus more closely. From the generic name we then are still able to conclude as to the appearance of the bacteria in question. In my later work on the lactic acid bacteria I have given nearly related cocci and rod-forms the same prefix (for instance, *Streptococcus* and *Streptobacterium*, *Betacoccus* and *Betabacterium*), which I think is also a practical arrangement. The prefix of the generic name ought no more than the specific name to allude to a person, not even to the person who first described the bacteria concerned; for this question is only of interest in the history of our science, but absolutely not from a natural-history point of view, and we ought not to encumber the bacteriologists of the future, who will have to handle thousands of bacterial species, with the history of each. The name of an organism ought to seem so natural to any one who is thoroughly acquainted with the organism and knows where it is to be found, that it will be nothing new to be remembered, but will serve on the contrary to associate his conception of the particular organism.

As to the family names of the bacteria, it will be convenient to let all of them end in *-Bacteriaceae*, by which it will be seen directly what is in question. If there are to be formed families of the cocci and spirilla, they must consequently be termed *Coccobacteriaceae* and *Spirillobacteriaceae* (or by the older name

of Zopf, *Spirobacteriaceae*). The reason why I have made an exception from this rule with the family *Actinomycetes* is because by the suffix -mycetes I wish to indicate that we have here the transition to the *Eumycetes;* but, in deference to the proposal of the Committee, I am willing to change the name to *Mycobacteriaceae*. On the other hand, I cannot agree with the Committee in following the old rule, that "a family name must be formed from one of its component genera with the suffix *aceae;*" for, if so, there would most frequently be no sense in the family name except in regard to this single genus. There cannot be any doubt but that we ought to form the family name in such a way that it denotes a property—and preferably the most characteristic one—common to all the bacteria which belong to the family in question. Accordingly, it is no improvement on the name, when for the family of oxidizing bacteria set up by me, the *Oxydobacteriaceae*, the Committee proposes the name *Nitrobacteriaceae*, which is quite misleading in respect to its first four genera.

The main objection of the Committee to my system is, that I do not pay due regard to priority. But what does that really mean? In old sciences such as zoölogy and botany we meet with really time-honored names, the legitimacy of which is quite indisputable; but in a new science like bacteriology we cannot consider the older names as anything more than provisional labels. Indeed, we have not advanced farther than to find a number of species, wherever we make a thorough-going study of a so-called bacterial species, and in by far the largest number of cases it is quite impossible to guess which of the new species is meant by the original author.

The Committee itself holds that we ought not to take into consideration the names dating from the time when microorganisms were not yet studied in pure culture—or rather the names proposed prior to 1885, when the system of Zopf appeared, the system which has formed the basis of the morphological classifications hitherto used. The Committee does not wish, however, to build up once more an exclusively morphological system, but a system based essentially on the far more important

biological properties, and as the researches initiated to that end are only in an embryonic state, it does not seem necessary to me that the bacteriologists out of regard for priority should relinquish all hope of establishing a practical nomenclature.

Although it is well-known that the red as well as the colorless sulphur bacteria may appear in all the forms known in the world of bacteria, and that even a single species of bacteria (for instance, *Crenothrix* and *Azotobacter*) in the first state of development may only divide in one plane but later in more planes, yet the Committee cannot admit that in case of other bacteria there may be a near relationship between sphere-, rod- and screw-forms. Still I entertain a perhaps not unwarranted hope, that my recently published monograph of the lactic acid bacteria may be able to change the opinion of the Committee. Here we have to do with a large group of bacteria consisting of sphere- as well as rod-forms, nevertheless forming a natural family which we could call *Lactobacteriaceae*. This family I have founded, of course, not only on the specially developed power of forming lactic acid (since there exist many different organisms which are able to form, at any rate, small quantities of lactic acid); but I base it upon the fact that the bacteria which we call true lactic acid bacteria have so many other properties in common that there cannot be any doubt about their close relationship. Thus, they are Gram-positive, faculatively anaerobic (without surface growth in stab culture), they make excessively great demands as to nitrogenous nutriment, and, most remarkably, in contradistinction to most other bacteria, they are unable to liberate oxygen from peroxide of hydrogen.

The sphere-forms belonging to the lactic acid bacteria ordinarily divide only in one plane, and, according as they form dextro- or laevo-lactic acid, they belong to the genera *Streptococcus* or *Betacoccus*. The acid-forming micrococci and sarcinæ, on the contrary, differ in so many respects from the true lactic acid bacteria, that they can hardly be placed here. Thus, my researches lead to the result that it is not the shape that makes the difference between sphere- and rod-formed bacteria, but, if anything, the division in one or more planes. The rod-forms

(the genera *Thermobacterium*, *Streptobacterium* and *Betabacterium*) belonging to the lactic acid bacteria are by no means always straight. They can be screw-formed and (especially *Bacterium bifidum*) bifurcated. Among the propionic acid bacteria, which also form a natural family, we once more meet with both sphere and rod forms, and among the latter very often club-shaped and forked forms.

I am glad to see that Breed, Conn and Baker (1918) in their critique of the report of the Committee agree with me in the view that "the shape of cell or form of body is not a fundamental character." It is so much the more strange that these investigators nevertheless finish by setting up a purely morphological system.

There is one further particular in which I must dissent from the Committee, and that is in setting up new genera of pathogenic bacteria (some of these being moreover named after persons), and in this particular too Breed, Conn and Baker agree with me. The pathogenic characters are not always so constant that they can be used as specific characters; they are often difficult to maintain when the bacteria are cultivated on artificial media. Non-pathogenic species can become pathogenic (for instance, certain streptococci and micrococci) through animal passages or through mixed infections, or (in the case of certain coli bacteria) simply by living in the intestinal canal. What is true of the parasites of animals, will certainly also apply to those of plants, and we thus know of moulds sometimes appearing as saprophytes, sometimes as parasites. Many so-called pathogenic species of bacteria ought more correctly to be considered as saprophytes from which more or less virulent varieties are readily developed, and although such species are more often met with in one genus than in another, we must be very cautious in setting up pathogenic genera. The interest which has been awakened in the pathogenic bacteria described in medical literature has hitherto left its trace in bacteriology to such an extent that it has been attempted to group all known bacteria around these. This is a step which must necessarily lead to the establishment of systems as artificial as if in the animal and vegetable kingdoms

we knew only the few parasitic species and tried to group all other animals and plants with them. The pathogenic bacteria are, fortunately, in the minority; the bulk of bacteria are leading a saprophytic existence and like the plants have their natural habitats in the soil. We therefore first have to put in order the saprophytes; then we can begin to mediate about where we have to place the parasites.

From the point of view here maintained I cannot follow Winslow in distributing the cocci firstly under the two groups parasites and saprophytes, and it seems to me that he is going rather too far when he uses the chromogenic property of the cocci to divide them into several genera. The formation of coloring matter can on an extreme estimate, and only when taken together with other characters, be adopted as a specific character; it is too variable to be used as a generic character. We must at times submit to being in doubt about what we are to call a species and what we are to regard as a variety; but the generic characters should be in some measure fixed, even though we must admit that the many transitional forms between the genera make it impossible to draw quite well-defined lines.

On the other hand, I have confirmed the correctness of the observation of Winslow that acid-forming cocci are always Gram-positive, whilst the non-acid-forming are, as a rule, Gram-negative, and consequently it is doubtless right on that basis to set up two groups of cocci, which—I suppose—belong in quite different places in the bacterial system. However, my two groups of cocci do not cover those of Winslow, as I believe I am warranted in separating the lactic acid-forming streptococci, and grouping them together with the rod-formed lactic acid bacteria. The acid-forming micrococci and sarcinæ I have brought together in the genus *Tetracoccus*, as I believe it to be quite as wrong to draw a limit between the micrococci and the sarcinæ as between the short- and long-chained streptococci. The property of cohering after division, though in a certain measure characteristic of the bacteria, is to a great extent influenced by the temperature and the composition of the nutritive matter. For the Gram-negative, non-acid-forming or, at most,

very slightly acid-forming cocci (among which must probably be reckoned the gonococci and the meningococci too, as well as Gram-negative streptococci, if such exist) we might simply use the generic name *Coccus*, or, if they should turn out to have *terminal* flagella, *Coccomonas*.

Even as unjustifiable as it would be to unite all the spherical bacteria into a great family, *Coccaceae*, would it be to set up the family *Spirillaceae*. The rule is indubitably that in every bacterial family we may meet with both sphere, rod and screw forms. Certainly the lophotrichic spirilla, both in regard to their morphological and to their biological properties, form a natural group. This thoroughly justifies the setting up of a genus, *Spirillum*, or better *Spiromonas*, a new designation, which would also make it possible to incorporate nearly related monotrichic species in this group. We should surely be warranted in doing so, since in other genera of cephalotrichic rods we meet with both monotrichic and lophotrichic species. The genus *Vibrio*, which the bacteriologists, one and all, reckon among the family *Spirillaceae*, can on the contrary scarcely be maintained, since these organisms pass gradually through the phosphorescent bacteria into the cephalotrichic rods.

The only morphological property of the bacteria which can perhaps be taken into account as a family character, is spore formation. Yet this property as such is not used in the case of the sarcinæ nor of the spirilla, and it is not always quite constant, even in the true bacilli. In cheese I have frequently met with aerobic, gelatin-liquefying, gas-producing plectridia which comparatively easily lost the ability to form spores and thus were not distinguishable from the Proteus bacteria. Thus, these interesting forms not only form the transition between the spore-forming and the non-spore-forming rods, but, as aerobic plectrida, between aerobic and anaerobic bacilli. In my opinion, we generally know too little as yet about the bacteria to be warranted in definitely setting up families, and I therefore consider we may safely put that off to the time when all the groups of bacteria have been as thoroughly studied as the lactic acid bacteria have recently been.

Buchanan (1917) sets up six orders of bacteria. I shall not undertake to discuss whether he is right or not, but only point out that if we follow him consistently the order *Eubacteriales* is necessarily to be divided into two orders, which we may call *Pseudomonadales* and *Peritrichinales*, as these two groups are by no means more closely related than *Pseudomonadales* and *Thiobacteriales* (the sulphur bacteria), forming together the cephalotrichic bacteria. Again to reduce the seven orders thus established to the corresponding families would perhaps not be a quite unsatisfactory solution of the family problem.

If we class together the genera which I have set up—with the amendments occasioned by my experience and that acquired by other researchers—into the above-mentioned two orders, we arrive at the following general synopsis:

Order 1: Pseudomonadales	*Order 2: Peritrichinales*
1 Methanomonas	1 Thermobacterium
2 Carboxydomonas	2 Streptobacterium
3 Hydrogenomonas	3 Streptococcus
4 Nitrosomonas	4 Betabacterium
5 Nitromonas	5 Betacoccus
6 Azotomonas	6 Propionibacterium*
7 Rhizomonas	7 Microbacterium*
8 Acetimonas	8 Tetracoccus
9 Fluormonas	9 Coccus
10 Photomonas	10 Bacterium*
11 Spiromonas	11 Bacillus*
	12 Clostridium*

As for the position of *Rhizomonas* (*Rhizobium*) in the system, I accept the proposal of the Committee, to place it next to *Azotomonas* (*Azotobacter*). I have myself really met with forked cells in different genera of bacteria and thus cannot attach a decisive, systematic importance to the furcation. After the researches of Barthel (1917) and those of Burrill and Hansen (1917) it must now be considered as certain that *Rhizomonas* is lophotrichic, and as a Gram-negative, lophotrichic nitrogen gatherer it ranks naturally with *Azotomonas*.

On the other hand, I cannot accept the proposition of the Committee to give the acetic acid bacteria the generic name

Mycoderma, since—apart from the circumstance that this name does not fit in with my nomenclature—it is already generally used as a generic name of certain pellicle-forming yeasts. Moreover, the designation *Mycoderma* (*Mycoderma vini* and *Mycoderma aceti*) dates from a far more ancient time than that cited by the Committee.

Although the property of setting free nitrogen from nitrates and nitrites is not of so general occurrence among the bacteria as is the property of reducing nitrate to nitrite or ammonia, we meet with the property of denitrification in different bacterial genera, and hence it would be unwarrantable to maintain the genera *Denitromonas* and *Denitrobacterium*, set up by me. As the property of liquefying gelatin also cannot be adopted as a generic character but only as a species character, I think it would be best to group together my earlier genera *Denitromonas* and *Liquidomonas* in a single genus, which can be conveniently termed *Fluormonas*, as the bacteria of this group are ordinarily fluorescent. I cannot agree to call this genus *Pseudomonas* merely out of regard for so-called priority, as each and all of the bacteria which belong to the order under consideration are really Pseudomonades as well.

As the phosphorescent bacteria form, biologically, a connected whole, I deem it correct to unite them so as to form one genus, for which a better name than *Photomonas* can hardly be found.

According to the experience we have acquired in regard to the nitrate-reducing bacteria there will scarcely be any reason to create a special genus for sulphate-reducing bacteria; but we naturally include the vibrios belonging here in the genus *Spiromonas*. The reason why I prefer this designation over the generic name *Spirillum*, has been mentioned above.

As for the second order of bacteria, I provisionally follow the proposition of the Committee with the differences justified by my researches on the lactic acid bacteria. I have no doubt that the genera marked with an asterisk (*), when studied more closely, will dissolve into two or more genera, some of which will cover some of those proposed in my natural bacterial system.

Whereas the shape of cells was formerly used as a family character, I have adopted it only as a generic one, and if we do not want to further confine its signification and only consider it as a specific character, we shall doubtless have to set up the genus *Propionicoccus* besides the genus *Propionibacterium*.

Microbacterium is to be understood as merely a provisional collective name for Gram-positive rods of size a little smaller than the ordinary bacteria. In biological respects some of these rods (*Bacillus acidophilus*) are closely related to the true lactic acid bacteria, whereas others approach the Tetracocci or the aerobic bacilli.

The genus *Tetracoccus*, including strictly aerobic as well as strictly anaerobic-species, is probably of as polygenetic a nature as is the genus *Microbacterium*, and the genus *Coccus* perhaps does not belong at all in the order of bacteria in question. In biological respects the Gram-negative, strictly aerobic, chromogenic cocci certainly appear to attach themselves rather closely to the genus *Fluormonas;* yet their place in the system cannot be determined with certainty until the arrangement of the flagella of the motile species has been studied.

The genus *Bacterium* will undoubtedly dissolve into several genera, of which I may especially mention the *Colibacterium* and *Aerogenesbacterium*. The reason why I am now inclined, in contrast to my earlier opinion, to consider the coli- and aerogenesbacteria as two different genera, is because they differ not only in morphological, but, as later researches have shown, also in biological respects. I have myself proved (1914), that the Aerogenesbacteria completely oxidize the carbohydrates when the nutrient matter offers a sufficient buffer effect, and they thus correspond with their name in forming more gas than do other bacteria, and Rogers, Clark and Davis (1914) have shown that in the gas developed by the Colibacteria there is proportionately more hydrogen than in that developed by the Aerogenesbacteria. Perhaps the Committee is right in not regarding the Proteus-bacteria (my genus *Liquidomonas*) as a separate genus, as their whole metabolism indicates that they are to be looked upon as gelatin-liquefying Coli- and Aerogenesbacteria.

Just as the behavior towards the different sugars is one of the most valuable characteristics of the acid-forming bacteria, so the relation to the different amino-acids can be used to divide the ammonia-forming bacteria, and this probably is the way to arrive at a closer division of the genus *Bacillus*.

The use of the term *Clostridium* as a generic name presents the inconvenience that under the same we must group together not only the clostridia but also the plectridia. The division into true butyric acid bacteria (*Butyriclostridium*), the requirements of which in regard to nitrogenous nutriment are very moderate (they are able to assimilate even the nitrogen of the air) and anaerobic, putrefying bacteria (*Putriclostridium*) seems natural to me.

As all Pseudomonades—so far as I know—are completely or partially decolorized by Gram, it is reasonable to seek a connection with the peritrichic bacteria among the Gram-negative representatives of the latter group, and it ranges naturally from the denitrificating species of the genus *Fluormonas* to the denitrificating species of the genus *Bacterium*. The development then from here has gone farther in one direction to the putrefying bacteria, characterized by breaking down amino-acids, and in the other direction to the lactic acid bacteria, which are not able to attack amino-acids, but demand the most complex nitrogenous nutriment.

REFERENCES

Barthel, C. 1917 Zeitschrift für Gärungsphysiologie, **6**, 13–17.

Breed, R. S., Conn, H. J., and Baker, J. C. 1918 Jour. Bact., **3**, 445–459.

Buchanan, R. E. 1917 Jour. Bact., **2**, 155–164, 347–350.

Burrill, T. J. and Hansen, R. 1917 Ill. Agr. Exp. Stat. Bull. 202.

Orla-Jensen, S. 1907 Det kgl. danske Videnskabernes Selskabs Oversigter, No. 5.

Orla-Jensen, S. 1909 Centralblatt f. Bacteriologie, 2 Abt., **22**, 305–346.

Orla-Jensen, S. 1914 International Dairy Congress at Bern.

Rogers, L. A., Clark, W. M., and Davis, B. J. 1914 Jour. Infect. Dis., **14**, 411–475.

Winslow, C.-E. A., and others 1917 Jour. Bact., **2**, 505–566.

VARIATIONS IN TYPHOID BACILLI[1]

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INTRODUCTION

Just as there is often great difficulty in diagnosing atypical clinical conditions, so great difficulty may be experienced in identifying bacteria which develop abnormal characteristics. The acquisition, by individual strains of many species of bacteria, of morphological and cultural characteristics which differ from the usual type has been noted by many observers and has been referred to more or less loosely by several different terms. Thus, Neisser (1906) and Massini (1907) used the word "mutation" to designate atypical forms of *Bact. coli*, Pringsheim (1911) speaks of an "adaptation" of bacteria, and Gurney-Dixon (1919) uses the term "transmutation."

We can sometimes follow such variations by gradual changes, from one stage to another, during which the bacteria pass through a process of evolution, adapting themselves to their surroundings.

Such variations may consist in the acquisition of new morphological, biochemical, or serological characters, in the loss of similar properties, or the two processes may occur at one and the same time. The change may be sudden or gradual, and is generally retained by the offspring.

The study of such variations is of fundamental importance to an understanding of the bacteria and may have considerable botanical importance since it would seem that processes of evolution or adaptation could be most easily investigated with

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forms whose characteristics are easily studied and in which generation follows generation with such speed that observation over the period of a year or longer might correspond to ages of development among the higher species. Moreover, from a purely practical point of view it is necessary to know to some extent just how much and how permanent a degree of variation is to be expected when well-known species are subjected to alternation between the conditions prevalent in artificial media and those existing in the human and animal bodies.

In the following studies the writer has occupied himself exclusively with the changes observed in the typhoid bacillus.

Strains

The cultures employed in this study were 138 in number and had been carried on artificial mediums since their isolation from patients. They were divided into three groups, as follows:

1. The stock cultures of the United States Army Medical School . . 116
2. Cultures collected by Dr. Oscar Teague 10
3. Cultures collected by Lieut. R. C. Colwell in France, and given us by courtesy of Lieutenant-Colonel Nichols 12

The sources of these cultures were as follows:

	strains
Blood cultures	55
Stool cultures	15
Urine cultures	11
Bile cultures	1
Sources unknown	56

Duration of cultivation on artificial media:

Over two years	15
One to two years	25
Six to twelve months	42
Less than six months	32
Recent isolation	11
Age unknown	13

Media and technique

In our experiments the media used and technique employed were as follows:

(a) Meat infusion broth. Meat infusion inoculated with *Bact. coli*, incubated about twenty-four hours at 37°C., autoclaved and filtered. To this was added 1 per cent pepton and 0.5 per cent sodium chloride.

(b) Nutrose broth. Instead of meat infusion 0.25 per cent nutrose was used. Both broth media were autoclaved for fifteen minutes at 15 pounds' pressure, and the reaction was adjusted to pH 7.0 or pH 7.1.

(c) Media containing sugars. In order to diminish the risk of decomposing the sugars during sterilization, they were dissolved in sterile distilled water, and heated in the autoclave for ten minutes at 10 pounds' pressure.

The sterilized sugar solution was added in the proportion of 1 per cent to the sterile broth together with 5 cc. of sterilized litmus or 5 cc. of 2 per cent phenol red and 1.2 cc. of decolorized 1 per cent aqueous solution of china blue (Morishima, 1917) per 100 cc. of the broth. Then the medium was transferred to small test tubes, and allowed to stand at least twenty-four hours at 37°C. in an incubator and for twenty-four hours at room temperature before being used.

For plates meat infusion agar (2 per cent) containing 1 per cent of the sugar was used. Decolorized china blue was then added in the proportion given above for the fluid medium. The reaction of all media mentioned above was adjusted to pH = 7.0 or pH = 7.1 by means of phenol red.

The stock cultures were transferred to agar slopes and incubated over night. Then pepton water tubes (1 per cent pepton 0.5 per cent salt solution, reaction pH 7.0) were inoculated from the slant cultures. After the latter had been incubated over night, one loopful of the pepton water growth was transferred to each tube of sugar medium; agglutination tests with the pepton cultures were also carried out.

In plating cultures on Endo or any other plates, one or two loopfuls of bacterial suspensions were usually streaked close to the margin of the Petri dish. The plate was divided into five parts by lines drawn on its bottom. From the first streak made with the loop, the suspension was spread over one-fifth of the surface; from the border of this area over the next third, and then from the last border over the remaining surface. By this method the distribution of bacteria was found to be satisfactory (Morishima, 1917). They were then kept in the incuba-

tor until the end of the experiment. Endo plates were occasionally inoculated from the sugar media in order to control possible contamination of the latter.

I. Variations in the Utilization of Carbohydrates

Variations in the biological behavior of the typhoid bacillus have been the subject of a great deal of investigation but, in the earlier work, especially, the identification of the races under observation was often incomplete (at least as far as one can judge from the publications) and all the reported results cannot be accepted without analysis.

Some of the earliest work was done on indol formation and on the fermentation of lactose.

Miss Peckham (1897) induced indol formation in a number of strains of *Bact. typhosum*.

Wilson (1902) isolated a strain from a typhoid carrier which produced acidity in lactose media at 22°C. while it did not produce it at 37°C. and the strain agglutinated only in 1:50 dilution of a typhoid serum of high titre. In other respects it resembled typical typhoid strains.

Klotz (1904) isolated from the St. Lawrence River water, a typhoid-like organism which he called *B. perturbans*. It fermented lactose and sucrose, formed indol, produced acidity in milk without coagulation and agglutinated with 1:2480 dilution of anti-typhoid serum.

McNaught (1905) isolated two organisms which he named *B. typhosus similans*, one of them from harbor water, the other from a well. The former did not produce indol and the latter did. Neither agglutinated in anti-typhoid serum. Both were motile when isolated but after some days of cultivation lost their motility.

Klotz's strain isolated from the water of the St. Lawrence River; McNaught's *B. typhosus similans;* and Wilson's strain isolated from a typhoid carrier's stool cannot be definitely accepted as real typhoid bacilli, because they were not sufficiently investigated to determine this fact positively.

Mandelbaum (1912) obtained a bacillus from the blood or feces of more than fifty patients with clinical typhoid fever in Munich, which he named *B. metatyphi*. This bacillus resembled *Bact. typhosum* in all respects except that it produced alkali instead of acid in media con-

taining glycerol. He showed that these cases were infected, in all probability from the same typhoid carrier, a woman who served as a milker in a dairy near Munich. This woman harbored both typical and atypical bacilli. The *B. metatyphi* retained the property of producing alkali in glycerol medium for five and one-half years when transplanted on plain nutrient agar. Russowici (1908) reported one case of *B. metatyphi*, and Ditthorn and Luerssen (1912) reported two similar cases.

Jacobsen obtained (1910) a bacillus which he described as *B. typhi mutabile* from a small epidemic of clinical typhoid fever in an insane asylum in Denmark. It resembled *Bact. typhosum* in all respects except the following:

1. It fermented mannitol after fifty hours. 2. Its growth was strongly inhibited on Conradi-Drigalski agar or plain agar which had been autoclaved. 3. Cultures from the plates showing retarded growth did not agglutinate in typhoid immune serum, but cultures of the same strain on media yielding good growth gave typical agglutination with typhoid immune serum and resembled *Bact. typhosum* in all other respects. *B. typhi mutabile* gave good specific agglutination five months after its isolation. There was normal growth on the Endo plates which removed the inhibitory action exerted on this strain by other media.

Fromme (1911) reports a bacillus, the growth of which was retarded on nutrient agar but which grew in ascitic fluid, human blood, guinea pig's blood, rabbit's blood and egg-yolk or on agar to which sodium sulphite had been added. His bacillus differed from Jacobsen's in that it agglutinated with typhoid immune serum from the start.

The variants of typhoid bacilli—*B. metatyphid* (Mandelbaum), *B. typhi mutabile* (Jacobsen) and the xylose non-fermenter of Weiss are unquestionably true *Bact. typhosum*.

Twort (1907) after growing a strain of *Bact. typhosum* for two years in lactose media succeeded in producing a strain that fermented lactose. He also conducted special experiments with a typhoid bacillus which had acquired the power of fermenting dulcitol. When such a culture was plated out on agar, subcultures from single colonies retained the dulcitol splitting powers, although they were still capable of being agglutinated by a typhoid immune serum, thus proving that the fermentation was not due to any contaminating microbe. On inoculating the dulcitol-fermenting typhoid culture into a guinea pig, subcultures were obtained showing the same reactions and these reactions were also maintained, even when the organism was grown for several

generations on ordinary pepton agar. His conclusions were that the sugar fermenting powers of an organism may be artificially changed by growing the said organism for a succession of generations in media containing a sugar which at the commencement of the experiment it was unable to ferment.

Kuwabara (1907, 1909) under the direction of Dr. Shiga isolated an atypical typhoid in addition to a typical one from a typhoid patient's stool. It fermented lactose, sucrose, and milk-whey as *Bact. coli* does and produced reddish color on Endo and Conradi-Drigalski plates but agglutinated in antityphoid rabbit serum in high dilutions just as a normal culture of typhoid bacilli did. After twelve to fifteen passages through plain laboratory nutrient media these atypical characters all disappeared.

The results of Twort and Kuwabara amount practically to a complete alteration of the identification characteristics of the typhoid bacillus. These observations are of the greatest theoretical importance but fortunately strains of this nature have been produced or observed so rarely that they cannot be regarded as a practically important factor of confusion in identification. This is apparent from Penfold's work cited below.

Penfold (1910a, 1910b, 1911, 1914) as cited by Dixon, working with twenty strains and carrying many of them for more than a year in a lactose medium, obtained only negative results; he showed that the Twort lactose fermenting strain gave rise to daughter colonies on lactose agar. This Twort culture fermented sorbitol in broth only after a number of days and Penfold found that it also gave rise to daughter colonies on sorbitol agar. He observed with some of his cultures late acid production in rhamnose broth and on transplanting from rhamnose broth to rhamnose broth after several weeks of incubation, he was able to obtain subcultures which fermented in one, two or three days. He also made a very careful study of the behavior of *Bact. typhosum* in dulcitol broth and on neutral red dulcitol agar. In one of his experiments in which fourteen strains were inoculated into dulcitol broth the first signs of acidity occurred in from five to fifteen days. If, after one month, subcultures were made in new dulcitol broth, an acid reaction was produced in from one to four days. Subcultures, which had been trained to ferment dulcitol rapidly showed great permanency; one such culture transplanted twenty-five times in pepton water during a period

of five months and then plated on neutral red dulcitol agar yielded only fermenting colonies. Twenty colonies from a MacConkey plate of pure typhoid were inoculated into dulcitol broth; the time required for acidity to appear varied from eleven to thirty-two days. Slow fermenters of dulcitol died in dulcitol broth in two months while quick fermenters remained alive longer. He found one strain which did not ferment arabinose, but which after three months' subculturings became a quick fermenter. Three strains that did not ferment glycerol became after eight months' subcultivation quick fermenters, but never in less time than three to four days. In plating out on glycerol media he found a mixture of quick and slow fermenters.

Reiner Müller (1908, 1911) did not observe acid production in rhamnose by any of his typhoid cultures and noticed that the colonies on the rhamnose plates remained small and delicate but that 5 per cent rhamnose produced no more inhibition than 0.5 per cent. He showed, further, that other bacteria of the typhoid-colon group are not inhibited by rhamnose. He observed no production of acidity by typhoid bacilli on litmus agar containing arabinose, dulcitol or raffinose, but he observed acidity on rhamnose Endo agar.

Bull and Pritchett (1916) found an atypical typhoid strain showing irregularity in fermentation, glucose, levulose, and dextrin being all positive and the other sugars negative; indol being positive as in the case of *Bact. coli*, but the organism agglutinated in 1:20,000 dilution.

Krumwiede, Kohn and Valentine (1918) inoculated thirty-seven strains of *Bact. typhosum* into xylose broth and found that twenty-nine produced acid in twenty-four hours while eight of the strains required from five to thirteen days for this result.

Winslow, Kligler, and Rothberg (1919) recently reported the results of similar investigations of various bacteria. They describe the typical typhoid bacillus as a Gram-negative, non-spore-forming rod, actively motile. It forms translucent irregular colonies on gelatin media and faint, nearly colorless growths on potato. It produces strong and prompt acid but no gas in media containing the hexoses, maltose, mannitol, sorbitol, xylose and dextrin; it does not attack arabinose, rhamnose, or lactose; produces a slight initial reddening of litmus milk, which after two weeks reverts to a neutral or slightly alkaline reaction. It

fails to form indol or liquefy gelatin, will not grow in asparagin-mannitol medium, does not reduce neutral red and causes browning of lead acetate media. It has low tolerance for acid, but rather high tolerance for brilliant green dyes and alkali. It has characteristic serum agglutination reactions and is found in human stools and urines as an actual or potential cause of typhoid fever.

OUR OWN WORK ON VARIATION IN THE UTILIZATION OF CARBOHYDRATES

Some of this work has already been reported in the Journal of Infectious Diseases, 1920, **26**, 52–76. The following table represents a condensed summary of previous work, together with new experiments performed since then:

Sugar fermentation

Arabinose broth

	Number of strains
First series. Meat infusion broth (litmus indicator)	114
Second series. Nutrose broth (phenol red, china blue, indicators)	117
Third series. The same medium	21
Fourth series. Meat infusion broth (phenol red, china blue, indicators)	24
Results were as follows:	
Positive on the second day	2
Positive on the third day	1
Positive on the fourth day	2
Positive on the sixth day	6
Positive on the seventh day	4
Positive on the ninth day	1
Positive on the tenth day	2
Positive on the eleventh day	1
Positive on the fourteenth day	1
Positive on the twenty-third day	1
Positive on the twenty-fourth day	1
Positive on the twenty-eighth day	1
Positive (total)	23
Negative on the 30th day	279

The proportion of positives is 8.24 per cent against 91.76 per cent of negatives.

Dulcitol

We could not obtain uniform fermentation results with this sugar, despite many attempts. Some strains gave rise to acid at one time and failed to do so at others. On the other hand certain strains gave rise to alkalinity at the first test and in later tests gave an acid reaction. Usually they produced acid in from one to three weeks.

	Number of strains
First series. Meat infusion broth (litmus)	115
Second series. Meat infusion broth (litmus)	57
Third series. Nutrose broth (phenol red, china blue)	21
Fourth series. Meat infusion broth (phenol red, china blue)	29
Fifth series. Meat infusion broth (phenol red, china blue)	139
The results were as below:	
Positive on the fourth day	1
Positive on the sixth day	26
Positive on the seventh day	15
Positive on the ninth day	31
Positive on the tenth day	2
Positive on the eleventh day	7
Positive on the twelfth day	11
Positive on the thirteenth day	9
Positive on the fourteenth day	11
Positive on the fifteenth day	11
Positive on the sixteenth day	3
Positive on the seventeenth day	3
Positive on the nineteenth day	1
Positive on the twenty-third day	12
Positive on the twenty-sixth day	3
Positive on the thirtieth day	9
Positive on the thirty-first day	1
Total positive on or before the thirty-first day	186
Negative on thirty-first day	175

The proportion of positives is 51.51 per cent against 48.49 per cent of negatives.

In all of these tests there was a greater tendency for the indicators (litmus, or china blue-phenol red) to become reduced than was evident in arabinose or xylose broth cultures. For this reason it is possible that the percentage of positives may be too low, as all tubes showing reduction were recorded as negative.

Glycerol

Glycerol broth

We prepared 1 per cent glycerol meat infusion broth, using china blue-phenol red indicator.

	strains
First series	123
Second series	10
Third series	10
Fourth series	10
Total	153

All of the cultures produced acidity.

Recently we studied these phenomena with 257 freshly isolated typhoid cultures from typhoid carriers' stools, using two different percentages of glycerol, namely 2 per cent and 6 per cent. We never found any alkali production in the early stage of incubation, but observed acid production between the third and eighth day; later some of the cultures showed reduction of color.

Inosite

Inosite is not a true sugar, but its character is similar to that of sugar and therefore it deserves discussion here.

Inosite broth

One hundred and forty-two strains were inoculated in 1 per cent inosite meat infusion broth, using china blue, phenol red indicator. No fermentation or gas production resulted after thirty days' incubation. Nine subcultures were transferred from inosite broth to inosite broth with the same result.

Raffinose

Raffinose broth

We used 1 per cent raffinose meat infusion broth, with china blue-phenol red indicator. In the first and fourth series, typhoid bacilli did not grow. The media showed slight acidity before

inoculation, but not sufficient to account for the inhibition of growth of typhoid bacilli. We do not know what substance caused this inhibitory action.

In the second series 60 strains and in the third series 21 strains were inoculated; all tubes became alkaline on the second day and showed no fermentation after thirty days' incubation. Of course both sets of media showed a neutral reaction before inoculation. Subcultures from a few of these raffinose broth cultures in raffinose broth again failed to produce fermentation.

Rhamnose

Rhamnose broth

One per cent rhamnose meat infusion broth, with china blue and phenol red as indicators was used for this experiment. One set of tubes was inoculated with 144 strains of typhoid, and another with 19 strains. Both produced alkalinity after twenty-four to forty-eight hours' incubation.

Salicin

Salicin broth

One per cent salicin meat infusion broth, containing china blue and phenol red indicator, was used. Two series of experiments, with 144 strains and with 21 strains were carried out, but no fermentation resulted during thirty days' incubation.

Xylose

We used for the most part 1 per cent xylose meat infusion broth, with china blue and phenol red indicator. During the course of this study we carried on many fermentation experiments, in which we found 8 per cent of 126 strains to be xylose non-fermenters, while the remaining strains all fermented xylose in twenty-four hours. Apparently these 10 negative strains, and in addition 12 strains brought from France by Lieutenant Colwell were found to be slow xylose fermenters. On carrying

subcultures of these strains from xylose broth to xylose broth ten strains (not Lieutenant Colwell's strains) became xylose fermenters in twenty-four hours (or somewhat later) after a few transfers.

CROSS FERMENTATION OF BACTERIA IN DIFFERENT SUGARS

It might be supposed that a single enzyme produced by bacteria when cultivated in sugar media, especially those closely related, such as xylose and arabinose, might ferment more than one sugar. To test this supposition, 6 xylose fermenters, 7 arabinose fermenters, 9 dulcitol fermenters (all fermenting in twenty-four hours) and 6 xylose slow fermenters, 6 arabinose slow fermenters and 7 dulcitol fermenters were inoculated into three sets of sugar media. We could not find any evidence of cross fermentation. After thirty-five days' incubation the fermenter of a given sugar still continued to ferment the same sugar, and no other. Therefore, the enzymes produced by bacteria are, as assumed by many workers, specific for each sugar.

DURATION OF THE FERMENTING POWER

Some strains, which had been artificially induced to ferment certain sugars, maintained their fermenting power after three or four months, or even half a year, although few transplantations were made from one fresh medium to another, this being done in some cases to media containing no sugar. Other strains, however, lost their fermenting power quickly, after only one or two transplantations. Dulcitol fermenters especially are apt to be changeable. Our records show two strains which at first fermented dulcitol in twenty-four hours. Later one of them fermented dulcitol only after four days and the other after twenty-one days. Similarly a strain which at first fermented arabinose in twenty-four hours, later required six days for the fermentation of this sugar.

FERMENTATION REACTIONS UNDER ANAEROBIC CONDITIONS

In the preceding experiments our cultures were grown under aerobic conditions. The following experiments were carried out anaerobically:

Two methods were used in these experiments. One was a combination of exhaustion and absorption with pyrogallol as used in this laboratory for routine work; and the other was the one devised by McIntosh and Fildes.

For the latter method we used a glass jar, connected by means of tubing with a tank of compressed hydrogen, which was inverted in a larger jar filled with water to a level of two or three inches above the inverted jar. The latter is held down by weights when its contents are displaced by hydrogen. Another tube fitted with a clamp connects this jar with the jar containing cultures. When everything is prepared in the culture jar a copper gauze package containing platinum asbestos is heated to redness and placed in the culture jar. The jar is sealed and the clamp released gradually thus allowing the hydrogen to enter the culture jar. The platinum asbestos will act as a catalyser to cause combination of oxygen and hydrogen.

When the oxygen in the jar is exhausted no more hydrogen will enter the jar. Then we seal the glass tubing to prevent the entry of air.

On account of its greater convenience we used the latter method more frequently.

Typhoid bacilli did not grow well under anaerobic conditions; consequently all reactions in sugar media were somewhat slower than under aerobic conditions.

A. Sugar broth cultures

We tried six strains in 0.01 per cent glucose, 1 per cent raffinose, 1 per cent arabinose, 1 per cent dulcitol, 1 and 2 per cent rhamnose, 1 per cent xylose and 2 per cent xylose. The results were as follows:

(a) *Glucose*

Aerobic: On twenty-four hours' incubation media distinctly showed alkalinity.

Anaerobic: Very slightly alkaline, even after ten days' incubation.

(b) *Dulcitol*

Aerobic: On tenth day, two cultures showed good acidity and on twentieth day four of them showed good acidity.

Anaerobic: Did not show acidity on tenth day; on twentieth day three of them showed slight acidity.

(c) *Rhamnose*

Aerobic: On the fourth day showed good alkalinity.

Anaerobic: On fifteenth day began to show alkalinity.

(d) *Xylose.*

True xylose fermenters ferment in twenty-four hours, under both conditions. The results with four slow fermenters follow:

Aerobic: On sixth day one of them and on the eighth day two strains began to ferment.

Anaerobic: Up to the twentieth day no change in reaction appeared, then the same strains that fermented xylose under aerobic conditions produced very slight acidity.

(e) *Arabinose*

Aerobic: On the third day, they showed alkalinity and on the fifth day one strain showed slight acidity.

Anaerobic: Slight alkalinity persisted until the twenty-first day.

(f) *Raffinose*

Aerobic: On second day showed alkalinity.

Anaerobic: On the eighteenth day showed slight alkalinity. In second series, we tested four strains, and in a third series, four strains on xylose broth but they did not show sufficiently definite changes to be described here.

It will be seen from the above that our own work does not in every way agree with the work of others cited at the beginning of this section. As stated above, we believe that the strains of Klotz, McNaught and Wilson cannot be definitely accepted as having been true typhoid bacilli.

In regard to Mandelbaum's *Bacillus metatyphi* we feel that our results seem to indicate that Mandelbaum was dealing with slow glycerol fermenters rather than with known fermenting strains. We should add, however, that we ourselves never encountered any strains which exhibited the characteristics described by Mandelbaum.

As for the strains of Jacobsen none of our cultures corresponded to these.

As to the results of Penfold and Reiner-Müller, our results agree pretty definitely with those reported by these workers.

No strains corresponding to those described by Bull and Pritchett were met with by us and we were able to find no similar ones described in the literature.

We have confirmed the observations of Krumwiede, Kohn and Valentine, and in addition have shown in this work, as well as in a previous publication with Dr. Teague, that rapid xylose fermenters can be produced from slow fermenters with considerable ease.

Although in almost every respect our work corresponds with that of Winslow, Kligler and Rothberg, they do not entirely correspond with the results of these workers in regard to the action of the typhoid bacillus upon arabinose. Subcultures that ferment arabinose rapidly still retain this characteristic after having been kept on plain nutrient agar for one or two months.

The enzyme produced by a typhoid bacillus from one of the sugars, xylose, arabinose, or dulcitol, may be greatly increased without affecting the production of ferments for the other two sugars. In anaerobic cultures of typhoid bacilli the lack of oxygen supply causes a partial inhibition of growth.

DAUGHTER COLONIES[2]

Reiner Müller (1908, 1911) first showed that *Bact. typhosum* produced daughter colonies on rhamnose agar. He examined a large number of cultures in this regard and found that they all gave rise to daughter colonies and further that *Bact. typhosum* produced daughter colonies in eight days on agar containing as little as 0.025 per cent of rhamnose and in fourteen days on agar containing only 0.01 per cent. He suggests that the development of daughter colonies on rhamnose agar might be utilized in the identification of *Bact. typhosum;* the results

[2] The term "daughter colony" is used throughout as signifying the type of secondary colonies arising spontaneously within the substance of the parent colony. The formation of these daughter colonies seems to signify that certain individual cells within the colony acquire the property of utilizing the sugar and therefore growing with much greater speed than the remaining bacteria making up the mother colony.

obtained by Penfold, Saisawa, and by us as far as they go, indicate that he was right in concluding that all typhoid cultures exhibit the phenomenon. Muller and Saisawa found that some other bacteria besides *Bact. typhosum* also give rise to daughter colonies on rhamnose agar.

Penfold (1911) found that the twenty strains of *Bact. typhosum* investigated by him all gave daughter colonies on rhamnose neutral red agar and he noticed acid production in none of the daughter colonies. But after a number of subcultures in rhamnose broth, he obtained a strain which fermented rhamnose. Such a rapid fermenter no longer produced daughter colonies on rhamnose agar and even when it was passed through thirteen generations of pepton water and plated on rhamnose agar it still did not give rise to daughter colonies. He found that the Twort lactose fermenting *Bact. typhosum* and a typhoid culture which had been trained to ferment dulcitol rapidly both produced daughter colonies on rhamnose agar. Three strains inoculated on neutral red dulcitol agar yielded daughter colonies as early as the third day and some of the latter were acid by the fifth day. Some plates showed as low as 2 per cent of colonies with daughter colonies, some as high as 50 per cent. Different plates inoculated with the same culture also showed variations within these limits.

Mandelbaum (1912) observed the production of daughter colonies from *B. metatyphi* on glycerol agar; from daughter colonies were obtained organisms which behaved in all respects like *Bact. typhosum*.

Bernhardt and Ornstein (1913) found colonies on dried agar with irregular outlines, and nucleus forms like anthrax colonies besides the normal typhoid colonies. On cultivating in bouillon this organism produced a film on the surface and was only slightly motile. They did not observe any phenomena of hypo- or in-agglutinability. These types we too have seen on dried plates but they are not real daughter colonies.

Gildermeister's (1913) typhoid strains produced daughter colonies on rhamnose agar except in one strain which had been isolated from a stool. The dysentery bacillus, Shiga-Kruse type and Strong type, *Bact. coli*, *Bact. alkaligenes*, paratyphoid bacilli, Gaertner bacilli, and cholera vibrios did not produce daughter colonies, but *Bact. dysenteriae*, Flexner type, and six out of fourteen strains of the "Y" type produced them. Six passages on ascitic agar or transplants over two weeks in rhamnose bouillon caused typhoid bacilli to grow colonies without daughter colonies on rhamnose agar.

Saisawa (1913) observed daughter colonies of *Bact. typhosum* when plated on rhamnose, dulcitol, arabinose (small ones) or erythrite agar after one week. Also he found daughter colonies on plating on rhamnose agar all of twenty-five strains of typhoid, three strains of Shiga type of dysentery, three strains of Flexner type, thirteen strains of Y-type, two strains of Strong type, two out of three strains of Pseudo-dysenteriae and none of ten strains of Paratyphoid B, five strains of Paratyphoid A, ten strains of Gaertner's bacillus, three strains of mouse-typhoid and six strains of *Bact. coli*. He could not obtain any variation of typhoid bacilli in culture media containing phenol or malachite green or caffein or by heating at 50°C. for one hour.

Daughter colonies have been observed and studied in connection with cultures of *B. anthracis*, *V. cholerae*, *Bact. coli*, *Bact. dysenteriae*, and other organisms, but they were either caused by sugars not considered in this paper or were not due to sugars at all.

OUR OWN WORK WITH DAUGHTER COLONIES

A. On sugar media without indicator[3]

On the second day or a little later, in an isolated thin colony, one or more very small heaped-up yellow or slightly brownish

[3] The sugars which we used for our work were analyzed at the Bureau of Standards, Department of Commerce, at Washington, D. C. The results were as follows:

	per cent	
Arabinose	94.1	
Moisture	1.1	Audubon Sugar School, Baton Rouge,
Insoluble matter	1.4	La., 62 grams arabinose
Undetermined	3.4	
Total impurity	4.8	
Rhamnose	89.6	
Moisture	9.9	Army Medical Museum, Rhamnose, AMS
Undetermined	0.5	
Xylose	100.6	
Moisture	0.2	257378, xylose, Difco Standard, Digestive Ferment Co., Detroit, Mich., U. S. A.
Raffinose	82.5	
Moisture	15.1	Raffinose—no label to show where from
Total impurity	2.4	
Dulcite		
Polarization	0	Dulcite 10 grams Special Chemicals Co.,
Polarization in presence of borax	0	Not inc., Highland Park, Ill.
Melting point	188	

yellow granules appear on the surface. Their borders can be distinctly seen by means of a hand lens or a low power microscope. Day by day they increase in thickness, in size, and in number, as the mother colonies enlarge. Later a confluent growth of the daughter colonies may entirely overgrow the mother colonies.

B. On sugar plates containing indicators

On plates which contain decolorized china blue the daughter colonies appear, blue in color and inside the mother colony. After a few days they increase in size, color, and number. The blue color doubtless is due to the production of acid by daughter colonies. Ten days or two weeks later, owing to a reduction of the dye, some of the fully grown colonies may have a brownish yellow color. The number of large blue daughter colonies that develop on the plate varies greatly according to the strains of typhoid bacilli employed, there being in some cases only 1 or 2, and in other cases 50 or 100 colonies.

Agglutination tests made with cultures of daughter colonies also showed no differences from those done with the original strain. When we used methylene blue eosin xylose plates (Holt-Harris and Teague, 1916) the mother colonies showed a pinkish color, but the daughter colonies appeared as white dots by transmitted light, some of which soon became black. These colonies exhibited the same rapidity of growth in the succeeding days that was described in the case of the blue daughter colonies on the china blue plates.

When we fished daughter colonies from the above plates and inoculated into the corresponding sugar broth, the latter showed acid production in twenty-four hours. We plated ten typhoid strains on eleven different 1 per cent sugar plates. On arabinose, dulcitol, raffinose, xylose and rhamnose plates from twenty to fifty strains were planted. The cultures were observed for about three weeks.

Below are described the variations in growth exhibited on each sugar medium:

1. Arabinose. Thirty-three strains plated on this medium. The daughter colonies appear in two to ten days, each colony usually containing many daughter colonies; a few of the daughter colonies frequently developed into large flat, deep blue colonies; all of the strains tested gave rise to daughter colonies.

2. Dextrin. No daughter colonies appeared.

3. Glucose. Colonies were smaller and denser than on other plates or control plates and no daughter colonies appeared.

4. Dulcitol. We plated forty cultures and daughter colonies appeared within two to five days. One or two opaque brownish yellow daughter colonies in a mother colony in succeeding days would grow so rapidly in size that sometimes the mother colony would be entirely covered. Furthermore, there is a tendency toward color reduction as the growth increases.

5. Galactose. Colonies were smaller than on control plain plates and no daughter colonies were produced.

6. Glycerol. We used 3 per cent glycerol plates for this purpose and obtained colonies which were very thick, opaque and yellowish brown in color. Plates containing China blue produced pale colonies on the first day, which became deep blue later. The color was reduced by the thick growth. No daughter colonies developed.

7. Inosite. No daughter colonies.

8. Lactose. No daughter colonies were seen.

9. Mannitol. No daughter colonies developed.

10. Maltose. There was good growth in point of size and thickness, but no daughter colonies were visible. Twenty strains were plated.

11. Raffinose. No daughter colonies developed, but ten days later, they showed papilliform colonies which did not increase in size nor in thickness on further incubation.

12. Rhamnose. All thirty typhoid strains which we tested on 1/10 per cent rhamnose plates gave rise to daughter colonies as well as on 1 per cent rhamnose plates. On the latter and on 2 per cent and 3 per cent plates, we could see many large opaque brownish yellow colonies scattered here and there with small daughter colonies and 1 per cent rhamnose Endo plates showed the same appearance.

The best method thus far developed for isolating typhoid bacilli from stools consists in plating upon a lactose medium containing brilliant green and an indicator for acid production. It seemed that a further improvement would be introduced by supplying a positive characteristic to the typhoid colonies instead of relying solely on the absence

of acid production. We attempted to accomplish this result by adding rhamnose (0.1 to 0.25 per cent) to brilliant green lactose agar, in the expectation that the daughter colonies within the typhoid colonies would furnish such a positive characteristic; however, we soon persuaded ourselves that this method has no practical value.

13. Salicin. No daughter colonies developed.

14. Sucrose. No daughter colonies developed.

15. Xylose. Fifty strains were planted. All xylose slow fermenters, thirteen in number, gave rise to daughter colonies on plates containing from 2 per cent to 0.04 per cent of xylose. The rapidly fermenting strains did not give rise to daughter colonies.

Sometimes xylose plates which contain 0.25 per cent glucose or which contained brilliant green eosin (Teague and Clurman) were used without interfering with the growth of the daughter colonies.

16. Control plain plates.

Some typhoid bacilli produced papillae-like forms on plain plates two or three weeks later when the plates were nearly dried up; such papillae sometimes occur also on plain plates inoculated with paratyphoid bacilli or *Bact. coli.* They never increase in size or in thickness. Therefore, we could not consider them true daughter colonies.

Under anaerobic conditions, we tested four strains, xylose slow fermenters (Rawling's, C-59, 57 and C-188) on 1 per cent xylose china blue plates; only two strains (C-59 and 57) produced daughter colonies on the eighth day; while under aerobic conditions, all four strains produced daughter colonies within three to five days.

RELATIONSHIP BETWEEN RAPID AND SLOW XYLOSE FERMENTERS

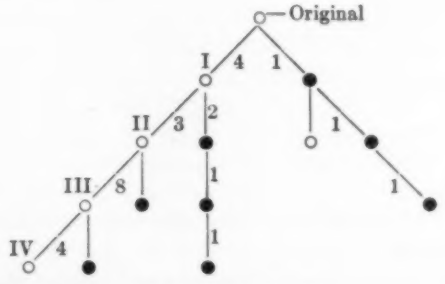
Kowalenko (1910, 1911) obtained *Bact. coli* cultures from Neisser, Massini (1907) and Burk, and tried to separate fermenters and non-fermenters from them after plating on Endo plates, and also from cultures which he isolated from a fever patient's stool on the Endo plate. He always obtained red-colored colonies after plating on Endo plates from a red-colored colony on the Endo plate, but he obtained white colonies and red colonies from a white colony on replating just as we observed above. He reached the conclusion after more study that mutation of bacteria could not be effected by influences from without,

by cultivation at various degrees of temperature, by long cultivation, by the addition of chemical substances, or by passing through the animal body. Saisawa (1913) tried in the same way to separate daughter colonies and mother colonies on plating typhoid bacilli but in vain.

It seemed to us that similar studies made with typhoid bacilli in regard to the separation of rapid and slow xylose fermenters from a single original strain might prove of great interest. Accordingly we made subcultures of two xylose slow fermenters two or three times on plain plates, each time fishing a single colony and planting from the single colony in 1 per cent pepton water. Then from the last suspension of a single colony we plated on 1 per cent xylose plates containing china blue or methylene blue-eosin. At the same time, a loopful of suspension was inoculated to 1 per cent xylose broth containing china blue indicator as a control. Repeated subcultures were made by this method in the hope that after some generations we might get a strain of non-xylose fermenters. We did not obtain such results, however. Charts of the subcultures obtained from the two strains follow:

Strain 57

This strain was always plated on 1 per cent xylose containing methylene blue and eosin.



● = quick xylose fermenter in twenty-four hours.
○ = slow fermenter.

Day on which acidity appeared in xylose broth

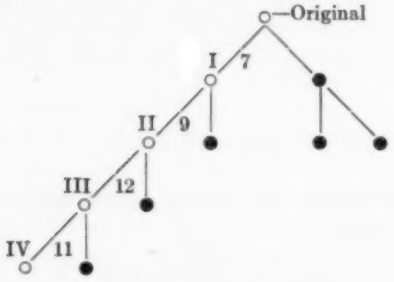
Original—fifth day
I—fourth day
II—fifth day
III—third day
IV—third day

Arabic numerals = interval in days between the appearance of a colony and subculture from it on 1 per cent xylose media containing indicator.
Roman numerals = number of generations.

We obtained only once a few pink colonies from a xylose fermenter. Thereafter we obtained only black colonies from xylose fermenter plates.

Rawling's

This strain was planted on 1 per cent xylose plates containing decolorized china blue.



Production of acidity

Original—seventh day
I—sixth day
II—fifth day
III—ninth day
IV—seventh day

We could not obtain any nonfermenter from the cultivation on xylose plates.

From quick fermenters which we had isolated slow and rapid types were readily obtained.

II. Variations in Reaction to Serum

Inagglutinability and agglutinability of typhoid bacilli

Agglutinability of typhoid bacilli isolated from specimens (blood, feces, urine, or bile) from the patient may vary greatly, depending to some extent on the number of culture generations for which they have been carried on artificial media. This has been reported by many workers (Förster (1897), Johnson and MacTaggart (1897), Müller, Eisenberg (1903), Sawyer (1912) and others). Thus, lack of agglutinability of the isolated bacilli in early culture generations is sometimes misleading in regard to diagnosis.

Schmidt (1903), for instance, erroneously reported typhoid bacilli as paratyphoid, owing to their inagglutinability. This inagglutinable state, acquired by the bacilli in the human body, can be easily produced by artificial means, such as cultivation on antityphoid serum broth. Such observations were first reported by Ransom and Kitashima, and by Müller. The former observed, in 1898, that the cholera spirillum lost its agglutinability when they cultivated it in anticholera serum, and the latter observed the same phenomenon in typhoid bacilli in 1903. The literature upon this subject is extensive and has been compiled in the articles of Eisenberg, Müller, and others.

As early as 1896 Metchnikoff and Bordet showed that cholera spirilla could partially lose their agglutinability under certain circumstances. Bail (1901) made similar observations with the typhoid bacillus, and Kirstein (1904) showed that cultivation at various temperatures could diminish the agglutinability of bacteria. It has been found, indeed, that organisms isolated from different cases of the same disease often varied considerably in their agglutinability in one and the same immune serum. This was noted by Grassberger and Schattenfroh (1900) in their studies upon anthrax. Bordet and Sleeswyk (1910) studying the whooping cough bacillus showed that when a horse is immunized with a whooping cough bacillus which has been grown upon blood media, the serum of this animal will powerfully agglutinate this strain, but possesses little or no agglutinating activity against the same strain habituated to growth on plain agar, an observation which they

interpret as meaning that the agar strain has lost its receptors for the absorption of the specific agglutinin and this inability to absorb agglutinin they, indeed, demonstrated by experiment. Park and his collaborators have studied these relationships particularly with the dysentery bacilli, and Park and Williams (1917) make the following statement:

"The maltose fermenting paradysentery bacillus of Flexner was grown on each of eleven consecutive days in fresh bouillon solutions of the serum from a horse immunized through repeated injections of the bacillus. The solutions used were 15, 4 and 1.5 per cent. The serum agglutinated the culture before its treatment in dilutions up to 1 to 800, and was strongly bactericidal in animals. After the eleven transfers the culture grown in the 15 per cent solution ceased to be agglutinated by the serum and ceased to absorb its specific agglutinins. The cultures grown in the 15 and 4 per cent dilutions of serum agglutinated well in dilutions up to 1 to 60 and 1 to 100, and continued to absorb agglutinins. The recovery of the capacity to be agglutinated was very slow, the cultures being transplanted from time to time on nutrient agar; after growth for sixteen weeks, during which it was transplanted forty-three times, it agglutinated in dilutions of 1 to 200. The culture grown in 4 per cent agglutinated in 1 to 500 dilution, and the one in 1.5 per cent in 1 to 800."

And in their new edition (seventh edition, (1920)) they say:

"The agglutinogenic power, or power to stimulate the production of other antibodies, is not lowered when bacteria become less agglutinable."

The presence of a capsule may interfere with or prevent agglutination. The capsule, developing best in body-fluid or tissues is probably a protective substance. Porges (1905a,b) has outlined a method for the removal of the capsule as a preliminary to agglutination.

Eisenberg (1913) studying a typhoid strain carried along in blood bouillon for a considerable period, found similar development of inagglutinability. And Schmidt (1903) has cited a case of a typhoid bacillus isolated from human disease in which inagglutinability led to prolonged error of diagnosis. Bail working with typhoid bacilli cultivated from the peritoneal exudate of infected guinea pigs showed that under such conditions the organism loses a considerable degree of its agglutinability and attributes this to the development of a capsule-like substance which insulates the bacteria against the antibodies.

A definite loss of agglutinability under similar circumstances was noted by Zinsser and Dwyer (1918) in connection with experiments

upon proteotoxin, but without their finding anything in the nature of a capsule which could explain the phenomenon. Ransom and Kitashima, Müller (1911), Hamburger, Walker (1904) and several other workers have also produced inagglutinability by cultivating on sera containing agglutinin. Porges and Prantschoff (1906) used this method, obtaining irregular results, and attribute this to individual variations in the strains used.

Moon (1911) produced two substrains of a single typhoid bacillus culture by the Barber method, one of which agglutinated with antityphoid serum and the other did not. A few generations later both bacilli showed equal agglutinability.

In Zinsser's book (1918) Infection and Resistance, he states: "This lessened susceptibility to antibodies is noticeable not only in strains cultivated from the body in disease, but can be produced artificially by cultivating the bacteria on inactivated, homologous immune serum. Such strains may not only increase in virulence, but lose in both agglutinability and susceptibility to bactericidal effects."

Sacquepee (1901) obtained similar variations by keeping the organisms in collodion sacs in the peritoneal cavity. Sawyer (1912) isolated a strain from a typhoid carrier's stool which did not agglutinate with a serum dilution of 1 to 50, but after 11 transplants within two weeks the culture became agglutinable. The same phenomenon has been observed by Scheller (1908).

Recently Gay and Claypole (1913) studying the typhoid bacillus, found that when they produced the carrier state in rabbits the organisms isolated from such rabbits (culturally true typhoid bacilli) failed completely to agglutinate in serum produced with a stock culture and which agglutinated such stock cultures in dilutions as high as 1:20,000. The blood and bile cultures which were inagglutinable by means of the ordinary antiserum, were readily clumped by means of a serum produced by immunizing rabbits with cultures grown on a blood agar medium. If confirmed, these observations, like those of Bordet and Sleeswyk, would indicate a complete alteration of antigenic properties by means of cultivation on blood media, and prolonged residence in the animal body; for the serum produced with the cultures upon blood, not only agglutinated these cultures, but also the plain agar growths, and if the cultures on blood were carried along for some generations on plain agar, they again became agglutinable by the serum produced with the plain agar culture. Gay (1914–15) uses this observation to explain the occasional inagglutinability in ordinary sera, of typhoid bacilli recently

isolated from human cases, and emphasizes the diagnostic difficulties which this may occasion. Bull and Pritchett (1916), however, repeating the work of Gay and Claypole found that 25 generations of cultivation in separate series upon plain agar and upon blood agar did not produce appreciable agglutination differences in a typhoid strain. They carried this out with 57 different strains of typhoid bacilli. Nichols too has contradicted the claim of Gay and Claypole that gall bladder infections could be regularly produced in rabbits by injection of typhoid strains grown on blood agar, an observation which would further strengthen the opinion of a fundamental change in reaction to the animal body and its fluids produced by cultivation upon blood constituents. Our own observations (in which we cultivate the typhoid strains upon normal rabbit's serum) also indicate that such a procedure does not exert any appreciable effect upon their agglutinability. Thus the results of Gay and Claypole concerning the inagglutinability of cultures obtained from infected human beings and rabbits would correspond in principle with the investigations of other workers. But the alterations obtained by them by simple growth upon normal blood agar cannot be accepted as conclusive in the light of contradictory results of Bull and Pritchett and of Nichols, and also in the light of our own failure to obtain appreciable changes in strains carried for many generations on normal rabbit serum broth.

It appears from these researches (and many others which might be cited) that the problem has not yet been solved in all particulars. But the general weight of evidence indicates that cultivation in the presence of specific serum antibodies alters the strains in the direction of lessened agglutinability.

The following experiments upon this phenomenon were carried out by us:

Materials used in agglutination tests

1. Broth. This consisted of 0.1 per cent Liebig's meat extract; 1 per cent pepton (Difco); and 0.5 per cent sodium chloride per liter; its reaction was adjusted to pH 7.0. It was sterilized in the autoclave at 15 pounds pressure for fifteen minutes.

2. Antityphoid serum from rabbits immunized against monovalent strain of typhoid bacilli (Rawlings, C-51, no. 3, Cohen, C-188). Each serum titre was 1:10,000 or 1:20,000 for our standard laboratory strains.

3. Normal rabbit serum. Serum obtained from normal rabbits and inactivated at 56°C. for half an hour. About 1 cc. of each medium was placed in small test tubes used for Wassermann work under strictly aseptic precautions, and kept in an ice-chest.

4. The strains used were plated on plain plates three times, and each time one single colony fished to 0.85 per cent sterile salt solution and from this plated on another new plate.

Technique

Stock cultures or newly isolated cultures as described above were suspended in 1 per cent pepton water, and transferred to serum broth or broth; thence retransferred from serum broth to new serum broth or from plain broth to new plain broth, by using a small platinum loop; then incubated at 37°C. Usually after twenty-four hours' growth the culture was plated on plain agar or implanted on slant, using a platinum needle or a very small platinum loop (in the use of the loop we took great precautions against including any serum broth); after standing overnight in the incubator at 37°C. the growth was evenly emulsified in 0.85 per cent salt solution (the bacterial growth always covered the entire surface of the slant). Special care was taken to have the emulsions of the cultures of as uniform a thickness as possible and for this purpose a tube of typhoid bacterial emulsion was kept for comparison.

Graded dilutions of the serum were made with 0.85 per cent sodium chloride solution and ranged from 1:50 to 1:24,300. Half a cubic centimeter of each dilution was transferred to small agglutination tubes and an equal amount of bacillary emulsion was added to each tube and also to a salt solution control.

The results were recorded after two hours' incubation at 37°C. and again after standing overnight in the ice-chest. The controls never showed agglutination. By this method cultures which had been grown on serum were allowed to develop for one generation on agar without serum before their agglutinability was tested. Controls were treated in the same way.

The serum media were occasionally tested for loss of agglutinating power, and were controlled for contamination by plating on plain plates or Endo plates or by inoculation in sugar media, but results were always negative. Such control cultures were made in sugar media (namely, xylose, arabinose, glucose, maltose, mannitol, lactose, sucrose,

rhamnose, raffinose, dulcitol) in broth, in litmus milk, and in 2 per cent glycerol; or plated on Endo plate; always, as stated above, with negative results.

STRAINS MADE INAGGLUTINABLE BY ARTIFICIAL MEANS

I. Antityphoid rabbit serum (Rawling's strain), with a titre of 1:10,000 for the homologous and for other laboratory strains, was mixed in proportions of one part of the serum to four parts of the broth in small test tubes. In the course of the experiments, this serum being exhausted, another antityphoid serum (made with strain C-51) with a titre of about 1:10,000 was substituted.

The same lot of broth was used in the controls.

The stock strains used were "Rawling's," "C-188," and "Cohen." These were prepared in the manner described above and inoculated into the media.

1. Rawling's strain. (Within thirty-eight days this strain was retransplanted twenty-four times from one tube to another, in two parallel series, one upon antityphoid serum broth, the other on plain broth.) During the first ten days, it was transplanted every day and later at intervals of several days. Agglutinability was tested eight times during this period with the same serum. Chart 1 shows the results.

As is shown by chart 1, (the power of agglutinability in dilution of 1:8000 fell to 1:300 after three days, and twenty-four days later, after fifteen transplants in fresh serum media, the agglutinability was recovered.) After that there was no remarkable difference between control and serum cultures, although the culture in serum broth was always somewhat lower in agglutinability than the control. The first readings of the serum broth culture after two hours' incubation always showed much lower agglutinability than the control cultures until the expiration of thirty-five days. The astonishing feature of this experiment was the fact that the serum strain seemed to recover its agglutinability after prolonged cultivation on serum, though at first it had lost it. For this reason, on and after the twenty-third day, we used

the other sera "C-51" and "3," for agglutination tests, but did not observe any striking difference between Rawling's serum and the other two sera in agglutinating Rawling's strain.

2. "C-188" strain was tested in the same way as the Rawling's strain.

Chart I

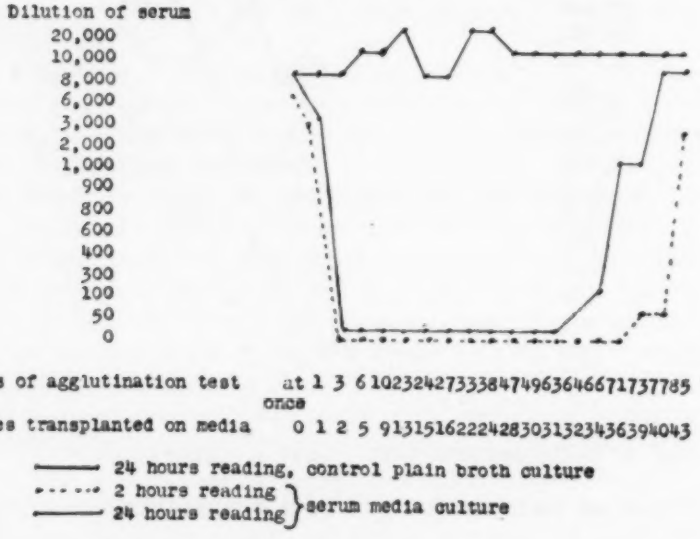
Rawling strain against Rawling's serum antityphoid

In this case, we made agglutination tests eleven times within sixty-three days. (Its agglutinability fell from 1:8000 to 1:3000 after one day's cultivation in serum media. Three days later agglutination occurred only in dilution of 1:100. Six days later,

it was below 1:50. On the twenty-seventh day it rose to 1:300 and after the thirty-fifth day to 1:20,000. Thereafter, from the thirty-fifth to the sixty-third day, which was the last day of this experiment, the serum strain and the broth control remained entirely parallel.) Other sera, C-51 and 3, were frequently used after the twenty-third day but no differences were seen between Rawling's serum and the other two sera.

Chart II

Cohen strain agglutination against Rawling's antityphoid serum



3. Cohen strain was tested in the same manner. We tested its agglutinability twenty-three times within eighty-five days, within which period we made forty-three transplantations. The results are shown on the next chart.

After seventy-one days this strain recovered its agglutinability gradually and at seventy-seven days it had almost completely recovered this property.) But readings two hours after incubation were always slightly lower than the twenty-four hours' readings. On the fourth day very slight agglutination in dilu-

tions of 1:50 and 1:100 occurred, visible only with a lens. After that not the slightest agglutination could be seen in any dilution up to 1:50 until the forty-ninth day. On the forty-ninth day slight agglutination was visible by lens up to 1:300 dilution, and on the sixty-fourth day, we could recognize good agglutination in 1:50 dilution of serum. Then within about two weeks it reached almost the highest dilution of the serum attained by the control culture. Nine tests were made with the Rawlings serum. On and after the twenty-third day, we tested its agglutinability five times with C-51 serum as well as with the Rawling's serum. Readings at the end of two hours showed no agglutination for the first four times but agglutination appeared in 1:100 dilution of the serum after the forty-fifth day. In twenty-four hours' readings no agglutination appeared in 1:50 dilution of serum until the twenty-seventh day while on the thirty-third day, and after, it occurred in a serum dilution of 1:900.

During the same period we tested the strain seven times with antityphoid serum (no. 3). Two hour readings showed that no agglutination appeared in 1:50 dilution of serum until the forty-ninth day. On the sixty-fourth day good agglutination appeared in 1:100 dilution. In twenty-four hours' readings until the thirty-third day no agglutination was seen in 1:50 dilution. From the thirty-seventh to the sixty-fourth day it always showed agglutination up to 1:300 dilution of serum.

Agglutinations of control cultures by each serum ranged from 1:8000 to 1:10,000.

In all experiments done with different sera (Rawling's, C-188, and Cohen) when we changed the serum media there was a slight tendency toward agglutination in the first generation on the new serum. After two or three generations in the new serum media this tendency disappeared.

II. We controlled our experiments with normal rabbit serum broth in place of antityphoid serum broth media. Four parts of broth and one part of normal rabbit serum (inactivated by heating at 56°C. for half an hour) were used for this purpose and a series of agglutination tests carried out with the same strains and in the same manner as before.

Agglutination tests of each culture were carried out five times within forty-three days, using Rawling's serum for the tests. Twenty-eight transplants were made during this period.

III. Since the experiments done so far seemed to show a delicate difference between the reactions of individual strains with different antityphoid sera, we decided to repeat them carrying a single typhoid strain both on an homologous serum and on an antityphoid serum produced with another stock strain. For this purpose we immunized two rabbits, in one case using no. 3 culture and in the other C-51. The former is a rapid xylose fermenter and the latter a slow xylose fermenter. We obtained sera which agglutinated our laboratory strains and their own specific strains in dilution of 1:15,000. Cultivation in normal serum in no case changed the agglutinability of the bacilli, which remained parallel in every way to that of the cultures carried on plain broth. Serum media was again prepared (one part serum to four parts broth) and tubed in small tubes. Experiments as described above were then carried out.

(1) Strain C-51. After two days' cultivation both in its own serum broth and in no. 3 serum broth, this strain slightly lost in agglutinability (1:8000). This continued for twelve days, but on the fifteenth day both series recovered agglutinability equal to that shown by the control cultures.

In no. 3 serum broth its agglutinability was lowered slightly more than by growth in the homologous C-51 serum broth, but there was no very considerable difference between them.

(2) Strain no. 3. This strain was tested in the same way as C-51, by cultivation in its own serum broth and in C-51 serum broth. On the third and seventh days inagglutinability on serum no. 3 was most marked (1:100) both for the culture carried on no. 3 serum and for the one carried on C-51 serum. Both gradually recovered agglutinability and reached normal agglutinability on the thirty-sixth day.

When we used C-51 serum for agglutination tests similar results were obtained. On the third day both series reached the maximum point (1:2000) of inagglutinability. Then they gradually recovered, although in this case recovery to normal agglutinability was delayed for twenty-two days.

This experiment showed that typhoid strains lose agglutinability in the same degree, whether cultivated in a serum broth of their own specific immune serum or on a serum immunized against other strains. No difference was observed between rapid xylose fermenters and slow xylose fermenters.

IV. The above experiments were performed with old stock cultures. We next repeated these tests using freshly isolated strains. Two strains (Owen and Boyle) which were obtained from typhoid carriers' stools were used after three generations on artificial media.

The media consisted of four parts of broth and one part of Rawling's or C-51 serum, the agglutinating titre being 1:15,000.

The Owen strain was cultivated in Rawling's serum and C-51 serum broth and transplanted into fresh media six times in seven days. It reached its maximum inagglutinability (1:900) in seven days with the same serum in which it was grown. When tested with the other serum its loss of agglutinability was not so great. A few days later both cultures in Rawling's serum broth and in C-51 serum broth recovered their agglutinability.

Observations were made with the Boyle strain in the same manner as with the Owen strain. In this case the maximum point of inagglutinability in Rawling's serum broth and in C-51 serum broth was reached on the fourth day.

On carrying out agglutination tests with Rawling's serum one strain (Rawling's serum culture) continued its inagglutinability (1:300) at least ten days, while the other culture in C-51 serum broth regained its agglutinability on the seventh day. The other tests with C-51 serum ran almost parallel. The maximum point of inagglutinability (1:900) was reached on the fourth day and agglutinability was recovered on the seventh day.

In the foregoing experiments observations were made concerning the character of the growth of typhoid bacilli in serum broth in vitro. During the first few days the bacteria grew like a mass of cotton at the bottom of the tubes; later when the bacteria had partly recovered agglutinability some organisms grew on the surface of the media, forming a film, as well as at the bottom. Up to this point the mass of bacteria is not easily broken up by shaking, but after recovery of agglutinability it becomes very

easy to break up the spongelike growth. A few days later the broth becomes turbid, at first, with a few clumps of agglutinated bacteria, and later only a uniform turbidity is present; in the last stage, at least one month later, the cultures grow slightly more turbid in the tubes, and about two months later, the general turbidity of the cultures is only slightly less marked than that of control cultures in broth.

The experiments just described were carried out in concentrated antityphoid serum media. It seemed important to observe what influence would be exerted on typhoid bacilli by cultivation in broth which contained a very low percentage of antityphoid serum. This might perhaps tell us what influence would be exerted upon the bacilli in the early stages of typhoid fever.

We prepared a number of broth tubes containing Rawling's serum in a proportion of 1:10,000. The titre of this serum against Rawling's culture was 1:10,000. We used two strains for these observations, one the Rawling's strain, the other a culture designated as Sanguist, which had been freshly isolated from a patient's blood.

We carried out agglutination tests eight times within thirty-three days during which we made twenty-two transplants. Rawling's strain showed practically no change, a result distinctly in contrast with its loss of agglutinability when grown in concentrated serum broth. The Sanguist strain diminished in agglutinability after three days although the difference between the serum culture and the control broth culture was not great. After thirty-two transplants thirty-three days later, it regained agglutinability completely. In short, in this experiment a small amount of antityphoid serum in media did not produce any marked change in agglutinability of typhoid bacilli.

When the serum contained blood cells the typhoid bacilli acted haemolytically and reduced the medium from a reddish color to yellow in twenty-four hours.

Several varieties of sugar media and litmus milk were inoculated from the cultures described above and no differences appeared between control cultures and serum broth cultures within three weeks.

AGGLUTININ ABSORPTION TESTS OF XYLOSE QUICK FERMENTER AND XYLOSE SLOW FERMENTER

The next experiments were undertaken to determine whether xylose quick fermenters and xylose slow fermenters differed in regard to serum reactions.

We immunized rabbits against the xylose fermenting strains "Cohen" and "no. 3," and against the xylose slow fermenters, C-51, Rawling, and C-188. The serum titre of each strain was between 1:10,000 and 1:15,000 for each strain. Three agar slants of xylose quick fermenter twenty-four hours old were prepared and a suspension in 5 cc. of 0.85 per cent salt solution made. To each suspension was added 5 cc. of each corresponding serum in dilution of 1:50. The tubes were then incubated for three hours at 37°C. After that, we centrifuged them for half an hour at high speed, and pipetted off the supernatant fluid. This was then made up with 0.85 per cent salt solution into a series of dilutions ranging from 1:100 to 1:16,200. To 0.5 cc. of each dilution was added 0.5 cc. of the suspension of a xylose quick fermenter, and similarly to another series of dilution, 0.5 cc. of a suspension of a xylose slow fermenter was added. The two series were incubated for two hours at 37°C., and allowed to stand overnight in the ice-chest. The results were read before placing in the ice-chest and again the next morning. As controls, agglutination tests were carried out using the original strains and fresh unabsorbed serums.

After absorption of both sera by slow xylose fermenters we found that practically all agglutinin had been removed. The only exception to this was a slight persistence of agglutinin in the tube containing the 1:200 dilution. Vice versa we absorbed the serum produced with xylose quick fermenters by means of a xylose slow fermenting organism and then looked for traces of agglutinin by adding suspensions of both xylose rapid fermenting and xylose slow fermenting strains. The results were the same as those obtained above.

Control tests gave negative results.

Xylose slow fermenters and xylose quick fermenters are therefore not serologically different.

It is worth noting in this connection that Lieut.-Col. H. J. Nichols tells us that he has found no difference between rapid and slow xylose fermenters in regard to their virulence for rabbits.

Having determined the peculiar conditions under which typhoid bacilli become inagglutinable and subsequently regain their agglutinability, without removal from the specific immune serum, it seemed important to determine whether this was due to inability to absorb the agglutinins or possibly whether it had some relationship to a changed reaction to electrolytes in solution. Also it seemed important to determine whether or not something analogous to an insulating capsule as described by Bail, Kuhnemann and others was responsible for the phenomenon.

Accordingly we first proceeded to carry out agglutinin absorption tests as follows:

a. Agglutinin absorption test

This test was carried out several times using the Cohen strain culture in serum broth for sixty-six days with 49 transplants in serum broth (see chart 2). As controls Cohen and C-188 strains were cultures in plain broth. The serum used was usually Rawling's, sometimes others. The technique employed was that described in the section on agglutinin absorption tests of xylose quick fermenter and xylose slow fermenter. The results follow:

TYPHOID STRAINS BEFORE TREATMENT BY THE SERUM	DILUTION OF RAWLING'S ANTITYPHOID SERUM								SALINE CONTROL
	100	200	400	800	1,600	4,800	14,150	43,200	
Cohen serum broth culture	−	−	−	−	−	−	−	−	−
Cohen broth culture (control)	+++	+++	+++	+++	+++	+++	++	−	−
C-188 broth culture (control)	+++	++	++	+++	+++	+++	+	−	−

+++ = complete agglutination.
++ = good agglutination.
+ = good agglutination but still cloudy.
− = negative. No agglutination.

Results are recorded after two hours incubation at 37°C. and standing in an ice chest over night. Hereafter we shall use the following abbreviations:

Cohen S = Cohen strain cultivated in serum broth
Cohen B = Cohen strain cultivated in broth (control)
C-188 B = C-188 strain cultivated in broth (control).

Tests done in Rawlings serum after absorption of this serum in dilution of 1:100 at 37°C. for three hours with the "serum" and the "broth" strains respectively:

(a)

BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM PREVIOUSLY TREATED BY COHEN S							SALINE CONTROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	−	−	−	−	−	−	−	−
Cohen B	+++	+++	+++	+++	+++	++	−	−
C-188 B	+++	+++	+++	+++	+++	+	−	−

(b)

BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM PREVIOUSLY TREATED BY COHEN B							SALINE CONTROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	−	−	−	−	−	−	−	−
Cohen B	+	−	−	−	−	−	−	−
C-188B	+	−	−	−	−	−	−	−

Tests with C-188 serum

Before absorption

BACTERIAL SUSPENSION	DILUTION OF C-188 ANTITYPHOID SERUM								SALINE CONTROL
	100	200	400	800	1,600	4,800	14,400	43,300	
Cohen S	−	−	−	−	−	−	−	−	−
Cohen B	+++	+++	+++	+++	+++	+++	+++	+	−
C-188 B	+++	+++	+++	+++	+++	+++	++	−	−

After absorption of C-188 serum in dilution of 1:100 at 37°C. for three hours.

(a)

BACTERIAL SUSPENSION	DILUTION OF C-188 SERUM PREVIOUSLY ABSORBED WITH COHEN S							SALINE CONTROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	−	−	−	−	−	−	−	−
Cohen B	+++	+++	+++	+++	+++	+++	+	−
C-188 B	+++	+++	+++	+++	+++	++	−	−

(b)

BACTERIAL SUSPENSION	DILUTION OF C-188 SERUM PREVIOUSLY ABSORBED WITH COHEN B							SALINE CONTROL
	200	400	800	1,600	3,200	9,600	28,800	
Cohen S	−	−	−	−	−	−	−	−
Cohen B	+	−	−	−	−	−	−	−
C-188 B	+	−	−	−	−	−	−	−

Tests with "Cohen" serum

Before absorption of serum

BACTERIAL SUSPENSION	DILUTION OF "COHEN" ANTITYPHOID SERUM								SALINE CONTROL
	200	400	800	1,600	3,200	6,400	12,800	23,600	
Cohen S	−	−	−	−	−	−	−	−	−
Cohen B	+++	+++	+++	+++	+++	+++	+++	+	−
C-188 B	+++	+++	+++	+++	+++	+++	++	+	−

After absorption of "Cohen" serum, diluted to 1:200 at 37°C. for three hours

(a)

BACTERIAL SUSPENSION	DILUTION OF "COHEN" SERUM WITH COHEN S							SALINE CONTROL
	400	800	1,600	3,200	6,400	12,800	25,600	
Cohen S	−	−	−	−	−	−	−	−
Cohen B	+++	+++	+++	+++	+++	−	−	−
C-188 B	+++	+++	+++	+++	++	+	−	−

(b)

BACTERIAL SUSPENSION	DILUTION OF "COHEN" SERUM WITH COHEN B							SALINE CONTROL
	400	800	1,600	3,200	6,400	12,800	25,600	
Cohen S	−	−	−	−	−	−	−	−
Cohen B	−	−	−	−	−	−	−	−
C-188 B	+*	−	−	−	−	−	−	−

* Slight.

These experiments showed that Cohen S cultures did not absorb the agglutinin of any of the three antityphoid sera.

The work of Bordet has demonstrated the essential importance of electrolytes in agglutination, and the studies of Neisser and Friedemann, Friedberger and others have shown that whereas small traces of salts will flocculate bacteria that have absorbed agglutinin, it requires very large amounts of the same electrolytes to flake out normal (insensitized) organisms. Since the researches of Neisser and Friedemann especially have shown that to a certain extent there is a relationship between the degree of sensitization and the amounts of salt necessary to bring about agglutination, we considered it of interest to determine by comparative tests the differences in sodium chloride concentration necessary to flake out, on the one hand, the Cohen serum strain, and on the other, the Cohen broth strain after both had been treated with homologous serum. This would show whether or not any trace of agglutinin had been absorbed by the Cohen S strain in the process.

Typhoid bacterial suspensions in Rawling's serum were centrifuged for thirty minutes and all the supernatant fluid decanted off for another agglutination test; the residue in both centrifuge tubes, one of them containing Cohen S and the other Cohen B strains was poured into a few centimeters of 0.85 per cent salt solution and without centrifuging but after slight shaking, all the fluid was decanted from the tubes, leaving a mass of bacteria. This was worked into an emulsion by means of a glass rod, and distilled water added drop by drop. The graded dilutions of the 20 per cent sodium chloride were prepared, to range from 20 per cent to 0.025 per cent. A half cubic centimeter of each dilution was transferred to small tubes and a half cubic centimeter of the above bacillary emulsion in distilled water was added to each tube and also to a distilled water control. The results were as follows:

The percentage shown below is the actual percentage after mixing salt solution and watery bacterial suspension:

	10	9	8	7	6	5	4	3	2	1	0.9	0.8	0.7	0.6	0.5	0.4	0.3	0.2	0.1	0.05	0.025	NO SALT
Cohen S emulsion	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Cohen B emulsion	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+				

This experiment shows that the Cohen S strain absorbed either no agglutinin at all or so little that it could not be demonstrated by strong solutions of sodium chloride.

We have now shown that the typhoid bacillus, which loses agglutinability after prolonged cultivation on immune serum, becomes inagglutinable because of its failure to absorb agglutinin. It was next desirable to determine, if possible, whether this inability to absorb antibody was due to an ectoplasmic insulation substance identical with or analogous to a capsule, since capsule formation in other bacteria has been shown to protect against serum effects. In spite of repeated attempts we never succeeded in demonstrating a capsule in our serum strains by staining methods. However, Bail and others have suggested that such capsular materials might be present in bacteria without showing demonstrable morphological change, except perhaps in the form of increased size of the bacterial cell as a whole. For this reason it seemed advisable to investigate this question by the method of Porges, who rendered the heavily capsulated and inagglutinable Friendländer bacilli agglutinable by dissolving off the capsule with weak acid and moderate heat.

If it be true that the inagglutinability of Cohen S is due to the formation of a capsule or something similar, then hydrolysis of protein of that capsule by Porges' (1905a, 1905b) method should restore the agglutinability. With this idea in mind, we tried the next experiment, using Rawling's serum, and carrying out the method of Porges exactly as described by him. The following tubes were prepared:

I. 3 cc. of each bacterial suspension plus 2 cc. of salt solution.

II. 3 cc. of each bacterial suspension plus 1 cc. of N/4 HCl plus 1 cc. of salt solution.

III. 3 cc. of each bacterial suspension plus 1 cc. of N/4 HCl plus 1 cc. of N/4 NaOH.

To 0.5 cc. of each of these mixtures graded serum dilutions from 1:100 to 1:25,800 were added; also to a salt solution control.

Results were as follows:

SOLUTION	BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM									SALT CONTROL
		100	200	400	800	1,600	3,200	6,400	12,800	25,600	
I	Cohen B	+++	+++	+++	+++	+++	+++	+++	+	−	−
	Cohen S	−	−	−	−	−	−	−	+S	−	−
II	Cohen B	−	−	−	−	−	−	−	−	−	−
	Cohen S	−	−	−	−	−	−	−	−	−	−
III	Cohen B	+S	+S	−	−	−	−	−	−	−	−
	Cohen S	−	−	−	+S	+	+	++	++	+++	+++

+S = slight agglutination.

In another experiment suspension I, II (without the addition of 1 cc. of salt solution) and III (without the addition of 1 cc. of N/4 NaOH solution) were made up. They were heated at 80°C. in a water bath for fifteen minutes, and then cooled in cold water. To no. II, 1 cc. of salt solution and to no. III, 1 cc. of N/4 NaOH solution were added respectively. After being shaken well, 0.5 cc. of each suspension was added to each of the serum dilutions described above.

The results were as follows:

SOLUTION	BACTERIAL SUSPENSION	DILUTION OF RAWLING'S SERUM									CONTROL
		100	200	400	800	1,600	3,200	6,400	12,800	28,600	
I	Cohen B	+	+	+	+	+	+S	−	−	−	−
	Cohen S	−	−	−	−	−	−	−	−	−	−
II	Cohen B	−	−	−	−	−	−	−	−	−	−
	Cohen S	−	−	−	−	−	−	−	−	−	−
III	Cohen B	+	−	−	−	−	−	−	−	−	−
	Cohen S	−	−	−	+S	+	++	++	+++	+++	+++

From these experiments it becomes apparent that the inagglutinability of our serum strains is not analogous to the similar inagglutinability of the Friedländer bacilli used by Porges in his experiments. At any rate our experiments do not permit us to conclude that the acquired inagglutinability of our strains is due to a capsule or to any analogous substance.

Acid agglutination

In 1911 Michaelis (1911) investigated the flocculation of bacteria by acids. His experiments were based upon the fact that serum globulins, casein, the so-called nucleoproteins and other forms of protein could be shown to precipitate at very definite optimum H-ion concentrations. Since Kraus (1897) and Neisser and Friedemann (1904) had claimed that the agglutination of bacteria depended upon the precipitation of a protein constituent of their cell bodies, Michaelis believed that for each species of bacteria an optimum H-ion concentration could be found which would agglutinate them. This he indeed demonstrated, and claimed that the typhoid bacillus especially could be shown to agglutinate at a definite H-ion concentration sufficiently characteristic to aid in the diagnosis of this organism. A number of investigators have confirmed this. For this reason we carried out the Michaelis technique with our broth and serum strains, to determine whether the H-ion optimum for agglutination had been altered by growth in serum.

The solutions were made as follows:

NUMBER	NORMAL SODIUM HYDRATE	NORMAL ACETIC ACID	STERILE WATER
	cc.	cc.	cc.
I	0.5	0.75	8.75
II	0.5	1.0	8.5
III	0.5	1.5	8.0
IV	0.5	2.5	7.0
V	0.5	4.5	5.0
VI	0.5	8.5	1.0
VII			10.0
VII	10 cc. of 0.85 per cent salt solution		

Both Cohen S and Cohen B cultures were suspended in sterile distilled water. Then 0.5 cc. of each of the above solutions and 0.5 cc. of bacterial suspension were put in small test tubes and the results recorded after two hours incubation at 37°C.

	I	II	III	IV	V	VI	VII	VIII
Cohen S	−	−	−	−	−	−	−	−
Cohen B	−	+S	++	++	+	+S	−	−
C-188 B	−	−	+	++	++	+S	−	−
Michaelis results of typhoid bacilli	−	+	++	++	+	−	−	−
Bact. coli by Michaelis results	−	−	−	−	−	−	−	−

This experiment shows that cultivation in serum has rendered the Cohen strain inagglutinable in an H-ion concentration which agglutinates the normal strain. In this test Cohen S resembles *Bact. coli*, but other tests (sugar fermentation) showed it to be an absolutely typical typhoid bacillus.

Similar tests were made with an inorganic acid, as follows:

HCl agglutination

Suspensions of both strains (Cohen S and Cohen B) were made in sterile water. From normal hydrochloric acid solutions in the following table were prepared by adding sterile water, 0.5 cc. of bacterial suspension, and 0.5 cc. of each acid solution were mixed in small test tubes and incubated at 37° for two hours, then stood in the ice chest overnight. The results were then noted.

The table below shows the final dilution of hydrochloric acid in the combination of acid and bacterial suspension:

	PROPORTION OF ACID											WATER CONTROL
	10	25	50	100	2 0	400	800	1,600	3,200	6,400	12,800	
Cohen S	−	−	+	++	+++	+++	+++	+++	+	−	−	−
Cohen B	−	−	−	−	−	+	++	+	−	−	−	−

Here Cohen S is very agglutinable in the presence of hydrochloric acid, although it is not so in the presence of acetic acid, as we have seen in Michaelis' test. Michaelis himself used only acetic acid.

SUMMARY AND CONCLUSIONS

In the study described above we have endeavored to investigate the variations which may occur in the characteristics of typical typhoid bacilli in regard to their abilities to utilize carbohydrates, and their behavior to serum antibodies under various conditions of cultivation. Underlying our plan of experimentation was the purpose of determining whether variations noted by others and described in many published accounts could properly be regarded as mutations in the botanical sense, and, secondarily, to contribute to the comprehension of the nature and the permanence of variations from type so often noticed in freshly isolated cultures, incidentally thereby shedding some light upon the practically difficult problems so often encountered by the bacteriologist in the identification of strains isolated from the human and animal body.

In order to obtain a true picture of the fermentative characteristics of a given species of bacterium it is clear from the foregoing study that the inoculation of a rather small number of strains into fluid media containing the various sugars and the recording of acid and gas production after a few days' incubation is entirely inadequate. A large number of strains must be investigated and the period of observation extended to thirty days, if necessary. Furthermore, it seems advisable to regard "fermentation" not as the production of gas and acid or even as the production of acid from the sugar by the bacterium, but simply as the utilization of the sugar as a food by the bacterium. Fermentation in this sense is demonstrated quite conclusively on agar plates if characteristic daughter colonies appear on the agar containing the sugar and no daughter colonies appear on control plates of the same agar without the sugar; precisely those cultures that yield unsatisfactory results in broth containing the sugar are apt to give rise to a well marked development of

daughter colonies. The writer believes that this method deserves a much more extended use in bacteriology than it has hitherto received. It seems likely that one could employ it to determine whether other substances besides sugars, for example, certain amino-acids, are utilized by the bacterium in question as a food, and in this way obtain a more accurate knowledge of the metabolism of the organism and possibly important diagnostic distinctions also.

By investigating a large number of strains of *Bact. typhosum*, using broth fluid and solid media and extending the period of observation over several weeks time it was observed that the behavior of many strains toward certain sugars varied widely from that of other strains. The slow fermenters of xylose resemble the mutations of the higher plants first described by de Vries, more closely than some of the other variants considered in this paper because they retain their characteristics quite constantly (for several years at least), so long as they are not grown in media containing xylose; all such strains investigated by us, with one exception, could be trained by long continued cultivation in xylose-media to produce acid in xylose broth in twenty-four hours like the typical *Bact. typhosum*. Furthermore, all of these slow fermenters, including the one that never produced acid in xylose-broth for us, showed daughter colonies on xylose agar; hence we were aware of the fact that these strains could utilize xylose as a food-stuff long before this had become evident from the observation of the xylose-broth tubes.

These observations would seem to indicate that even in the slowest xylose fermenters the xylose utilizing power is potentially retained as a latent characteristic. This would prevent our correctly applying to such cultures the term "mutation" in the sense of de Vries. Working with bacteria we are enabled to observe in a short time a sequence of generations far beyond anything that can be observed with higher plants and our work suggests, though of course in an entirely inconclusive way, that at least some of the "mutations" described by botanists may represent, in fact, a suppression of characteristics which remain latent and might easily become apparent again could a sufficient

number of generations be subjected to an environment in which this characteristic could again become useful.

The inoculation of solid media proved that all of our strains of *Bact. typhosum* utilize both arabinose and dulcitol for their nutrition. In fluid medium nearly half of the strains produced acid in dulcitol in from five to twenty days while only a small percentage showed acid production from arabinose. In striking contrast to the behavior of the typhoid strains in xylose broth, the strains that produced acid in arabinose or dulcitol in one test often failed to produce acid when the test was repeated under similar conditions.

All of our typhoid strains gave rise to daughter colonies on rhamnose agar, but none were observed to produce acid in rhamnose broth.

Our cultures showed no daughter colonies on raffinose agar and did not produce acid in raffinose broth. It is thus apparent that instead of stating that a given species of bacterium ferments such and such sugars, it should be said; that the organism in question can utilize such and such sugars as a food; that the organism produces acid in twenty-four hours in certain of these sugars; that a majority of the strains produce acid after several days in such and such sugars; etc., etc. Having obtained this information concerning certain related species of bacteria it should usually be easy to select those sugars which would yield the most reliable information for the differentiation of the species. Thus, Krumwiede (1918), and quite recently Jordan also, have recommended acid production in rhamnose broth as the most fundamental method of differentiating the paratyphoids from *Bact. typhosum*. We agree that this is a good practical medium for the differentiation of these species but we should bear in mind that *Bact. typhosum*, although it does not show acid production in rhamnose broth, is nevertheless able to utilize this sugar as a food.

Considerable discussion has arisen concerning the question whether certain of the variations from the normal type mentioned in this paper represent true mutations in the sense of de Vries. The writer believes that this term, which defined changes of a

definite character occurring in higher plants should not be introduced into bacteriology; for the bacteriologist, who studies his species not only from the morphological point of view, but also with regard to biochemical and immunological reactions, and who observes not a few generations only, but hundreds and thousands of generations, would almost surely have to modify the conception of the term in such a manner as to cause confusion to the botanist. It, therefore, seems advisable to leave the term mutation to the botanists and, for the present at least, to speak of atypical varieties of bacteria or simply of variants.

Typhoid bacilli grown upon normal serum do not become inagglutinable. Cultivated continuously upon specific immune serum they at first become inagglutinable, but if such cultivation is persisted in for two weeks longer, eventually these strains again become agglutinable. In some cases this return to normal agglutinability does not occur until the seventy-second day. This, however, is exceptional. It is important to notice that, whereas in other experiments the normal characteristics had developed, in this case a return to normal reaction with serum was brought about in spite of a continuous subjection to the unusual environment.

Inagglutinability of the typhoid bacillus is accompanied by inability to absorb agglutinin. There is no difference whatever in these relations between the xylose slow and xylose rapid fermenters. Our experiments do not indicate that capsular material is concerned in the inagglutinability. Treatment by the Porges method does not render the inagglutinable strains agglutinable.

Acid agglutination experiments by the method of Michaelis showed that the inagglutinable serum strain reacted negatively in the increasing concentrations of acetic acid, being in this way similar to colon bacilli, whereas the same strain cultivated in broth reacted with the acid typically as did Michaelis typhoid cultures. In other words, the inagglutinable strains showed a changed reaction in regard to hydrogen ion concentration of the environment.

Finally, we believe that since all the alterations brought about by artificial environment in the typhoid bacillus were rapidly lost when the organisms were returned to the environments prevailing under the usual cultural conditions and in the case of the inagglutinable strains, even in the course of persistent abnormal environment, the changes observed by others as well as by us should properly be regarded as variants and cannot be spoken of with accuracy as mutations in the sense of de Vries.

REFERENCES

BAIL 1901 Prager Med. Woch., Nr. 7.
BERNHARDT AND ORNSTEIN 1913 Berl. klin. Woch., Nr. I, 1519.
BORDET AND SLEEWIJIK 1910 Ann. de l'Inst. Pasteur, **24**, 476.
BULL AND PRITCHETT 1916 Jour. of Exp. Med., **24**, 55
EISENBERG, P. 1903 Cent. f. Bakt., **34.**
EISENBERG, P. 1913 Ergeb. der Immunitäts. Exper. Ther. Bakt. u. Hyg., 73.
FORSTER 1897 Zeitsch. f. Hyg., Bd. 24.
FROMME 1911 Cent. f. Bakt. 1 Abt. Orig., **58**, 445.
GAY, FREDERICK P. 1914–15 The Harvey Lectures, p. 49.
GAY AND CLAYPOLE 1913 Arch. of Intern. Med., **12**, 614.
GILDERMEISTER, E. 1913 Arbeit a. d. k. Gesundh. Amte, **45**, 226.
GURNEY-DIXON S. 1919 The Transmutation of Bacteria
HOLT-HARRIS AND TEAGUE 1916 Jour. of Infec. Dis., **18**, 596.
JACOBSEN, K. A. 1910 Cent. f. Bakt. Orig., **56**, 208.
JOHNSTON AND MCTAGGART 1897 Montreal Med. Jour.
KIRSTEIN 1904 Zeit. f. Hyg., **46**, 229.
KLOTZ, OSCAR 1904 Jour. of Med. Res., **11**, 475.
KOWALENKO, A. 1910 Zeitsch. f. Hyg. u. Infekt., **66**, 277.
KOWALENKO, A. 1911 Cent. f. Bakt., 1 Abt. Ref. **48**, 658.
KRAUS, R. 1897 Wien, klin. Woch., Nr. 32.
KRUMWIEDE, COHN AND VALENTINE 1918 Jour. of Med. Res., **38**, 89.
KUWABARA, CH. 1907 Saikingakuzassi, No. 143.
KUWABARA, CH. 1909 Cent. f. Bakt. Ref. 1 Abt. **42**, 135.
LUERSSEN AND DITTHORN 1912 Cent. f. Bakt., 1 Abt. **58**, 47.
MANDELBAUM, M. 1912 Cent. f. Bakt. 1 Abt. Orig. **63**, 46.
MASSINI 1907 Arch. f. Hyg. **61**, 251.
MCNAUGHT, J. G. 1905 Jour. of Path. and Bact., **10**, 380.
MICHAELIS, LEONOR 1911 Deut. med. Woch., 969.
MOON, V. H. 1911 Jour. of Inf. Dis., **8**, 463.
MORISHIMA, K., AND TEAGUE, OSCAR 1917 Jour. of Inf. Dis., **21**, 145.
MÜLLER, REINER 1908 Deut. Med. Woch., 1961.
MÜLLER, REINER 1911 Cent. f. Bakt. 1 Abt. Orig. **58**, 97.
MÜLLER, PAUL TH. 1903 Münch. med. Woch, **1**, 56.
NEISSER, I. M. 1906 Cent. f. Bakt. 1 Abt. Ref. **38**, 98.

NEISSER AND FRIEDEMANN 1904 Münch. med. Woch., **51**, 465 and 827.
PARK AND WILLIAMS 1917 Pathogenic Microörganisms, 6th Ed., 208.
PARK AND WILLIAMS 1920 Pathogenic Microörganisms, 7th Ed., 55.
PECKHAM, A. W. 1897 Jour. of Exper. Med., **2**, 549.
PENFOLD, W. J. 1910 a Brit. Med. Jour., **2**, 1672.
PENFOLD, W. J. 1910 b Jour. of Path. and Bact., **14**, 406.
PENFOLD, W. J. 1911 Jour. of Hyg., **11**, 329.
PENFOLD, W. J. 1914 Brit. Med. Jour., **2**, 710.
PORGES, OTTO 1905 a Wien. klin. Woch., 691.
PORGES, OTTO 1905 b Zeit. f. Exp. Path. u. Ther.
PORGES AND PRANTSCHOFF 1906 Cent. f. Bakt., **41.**
PRINGSHEIM, HANS 1911 Med. Klinik, **1**, 144
RUSSOWICI 1908 Münch. med. Woch., **55**, 2507.
SACQUEPEE 1901 Ann. de l'Inst. Pasteur, **15**, 261.
SAISAWA, KOHZOH 1913 Zeit. f. Hyg., **74**, 61.
SAWYER, WILBUR A. 1912 Jour. A. M. A., **58**, 1336.
SCHATTENFROH, A., AND GRASSBERGER, R. 1900 Arch. f. Hyg.
SCHELLER, ROBERT 1908 Cent. f. Bakt. **46**, 385.
SCHMIDT 1903 Wien. klin. Woch., 873.
TWORT, F. W. 1907 Proc. of Royal Soc. of London, Ser. B., **79**, 329.
WALKER, AINLEY AND MURRAY 1904 Brit. Med. Jour., **2**, 16.
WILSON, W. JAMES 1902 Brit. Med. Jour., **12**, 1910.
WINSLOW, KLIGLER AND ROTHBERG 1919 Jour. Bact., **4**, 428.
ZINSSER, HANS 1918 Infection and Resistance, 2 Ed., Chap. 9.

SOLID CULTURE MEDIA WITH A WIDE RANGE OF HYDROGEN OR HYDROXYL ION CONCENTRATION

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A considerable number of investigations, made during the past few years, have extended our knowledge of the profound influence exerted by acids and alkalis upon the growth of micro-örganisms. This is especially true in the case of such fungi and bacteria as lend themselves readily to cultivation on artificial media. Investigations have also clarified many problems related to these media themselves, such as the influence of acids and alkalis on colloidal hydration and jellification, the buffer action of proteins and salts, the devising of improved colorimetric and electrometric technic for the measurement of hydrogen and hydroxyl ion concentration, etc. The point covered in the present study, which grew out of an attempt to determine the limit of tolerance of certain organisms to acid and alkali on solid media, does not appear to have been brought out in any foregoing investigation. In previous studies use has been made of liquid media for very tolerant organisms, even for forms which are known to thrive best on solid media, since it has been impossible to make agar or gelatin with high pH values solid. It is the present purpose, therefore, to show that acids and alkalis need not materially modify the physical properties of agar and gelatin media within, and even far beyond, the limits of tolerance of any living organism.

METHODS

The media were prepared by adding either 1 or 2 per cent commercial agar or 10 or 15 per cent bacto-gelatin to a bouillon consisting of 0.3 per cent Liebig's beef extract, 1 per cent Armour's

peptone and 0.5 per cent sodium chlorid. They were then heated in an autoclave, flasked, and sterilized, for fifteen minutes at 15 pounds pressure in the case of agar, and 10 pounds pressure in the case of gelatin. No attempt was made to adjust the reaction of the media to neutrality prior to sterilization. The acid used was hydrochloric, with a specific gravity of 1.20 or it possessed an HCl concentration of 39.11 per cent. The sodium hydroxid had a specific gravity of 1.226 or an NaOH concentration of approximately 20 per cent. Strong acid and alkali were employed to eliminate the factor of dilution of the media. Upon removal from the autoclave the agar was cooled to about 50°C. and the gelatin to about 40°C. before the addition of appropriate quantities of acid or alkali, and were maintained at these temperatures while 10 cc. portions were withdrawn with a pipette and put into test tubes. The acid or alkali was added to these 10 cc. portions with a 1 cc. pipette graduated in tenths. After the addition of the acid or alkali the tubes were well agitated and were further cooled with the results indicated in the tabulations which follow.

EXPERIMENTAL

Only those proportions of agar or gelatin which are commonly employed in making culture media were used in this study but they indicate, as would be anticipated, that the jellifying power is modified by the proportion of colloidal material added. The results with 1 and 2 per cent agar are shown in table 1.

It will be noted that the limits of solidification of 1 per cent agar are approximately 5.11 per cent acid and 0.39 per cent alkali whereas 2 per cent agar does not lose its jellifying power until 6.51 per cent acid or 0.58 per cent alkali has been added. A better appreciation of the degree of acidity and alkalinity of these limits can be gained when they are compared with pH values determined by the colorimetric method of Clark and Lubs (1917). It was found that the addition of 0.1 cc. of HCl to 10 cc. of agar gave a concentration of about pH 1.4 and 0.03 cc. NaOH a concentration of about pH 9.2. In reaching the limits

in 2 per cent agar it will be seen that 20 times this volume of acid and 9 times this volume of alkali were employed. Manifestly these limits are only approximate and could be more accurately determined by improved technic. They are, however, far beyond the limits of tolerance of microörganisms and are intended only to show that hydrogen or hydroxyl ion concentration need not be limiting factors in the preparation of solid agar media.

TABLE 1

Effect of acid and alkali on solidification of agar

1 PER CENT AGAR						2 PER CENT AGAR					
Agar	HCl		NaOH		Physical state, 20°C.	Agar	HCl		NaOH		Physical state, 20°C.
cc.	*cc.*	*per cent*	*cc.*	*per cent*		*cc.*	*cc.*	*per cent*	*cc.*	*per cent*	
						10	2.0	6.51			Semisolid
						10	1.7	5.68			Solid
10	1.5	5.11			Semisolid	10	1.5	5.11			Solid
10	1.2	4.19			Solid	10	1.2	4.19			Solid
10	1.0	3.55			Solid	10	1.0	3.55			Solid
10	0.7	2.55			Solid	10	0.7	2.55			Solid
10	0.5	1.86			Solid	10	0.5	1.86			Solid
10	0.3	1.14			Solid	10	0.3	1.14			Solid
10	0.1	0.38			Solid	10	0.1	0.38			Solid
10			0.1	0.19	Solid	10			0.1	0.19	Solid
10			0.2	0.39	Semisolid	10			0.2	0.39	Solid
10			0.3	0.58	Liquid	10			0.3	0.58	Semisolid

All of the agar media in these series appear to be able to remain solid for an indefinite period when they are maintained at room temperature. If, however, they are autoclaved and then cooled all of the acid media were found to remain liquid, whereas the tubes of 1 per cent agar with 0.1 cc. NaOH and 2 per cent agar with 0.1 and 0.2 cc. NaOH became solid again.

Considerably larger amounts of acid or alkali must be added to 10 and 15 per cent gelatin to destroy the jellifying power as shown in table 2.

In the case of 10 per cent gelatin the limits are seen to be about 9.02 per cent HCl and between 3.33 and 4 per cent NaOH, and of 15 per cent gelatin between 9.02 and 10.15 per cent HCl

and about 4 per cent NaOH. When these media were examined after having been maintained in an ice chest at about 10°C. for twelve hours, all of those to which more than 1 cc. of NaOH had been added were found to have become liquid, and a heavy whitish precipitate had formed. All of the tubes to which HCl had been added were still solid, however. All of the tubes containing media which had remained solid were placed in boiling water until the media had liquified whereupon they

TABLE 2

Effect of acid and alkali on solidification of gelatin

10 PER CENT GELATIN						15 PER CENT GELATIN					
Gelatin	HCl		NaOH		Physical state 7.5°C.	Gelatin	HCl		NaOH		Physical state 7.5°C.
cc.	cc.	per cent	cc.	per cent		cc.	cc.	per cent	cc.	per cent	
						10	3.5	10.15			Liquid
10	3.0	9.02			Semisolid	10	3.0	9.02			Semisolid
10	2.5	7.82			Solid	10	2.5	7.82			Solid
10	2.0	6.51			Solid	10	2.0	6.51			Solid
10	1.7	5.68			Solid	10	1.7	5.68			Solid
10	1.5	5.11			Solid	10	1.5	5.11			Solid
10	1.2	4.19			Solid	10	1.2	4.19			Solid
10	1.0	3.55			Solid	10	1.0	3.55			Solid
10	0.5	1.86			Solid	10	0.5	1.86			Solid
10			0.5	0.95	Solid	10			0.5	0.95	Solid
10			1.0	1.82	Solid	10			1.0	1.82	Solid
10			1.2	2.14	Solid	10			1.2	2.14	Solid
10			1.5	2.61	Solid	10			1.5	2.61	Solid
10			1.7	2.90	Solid	10			1.7	2.90	Solid
10			2.0	3.33	Solid	10			2.0	3.33	Solid
10			2.5	4.00	Liquid	10			2.5	4.00	Semisolid

were again cooled to 7.5°C. The alkaline gelatin again solidified, but 1.5 cc. of HCl in 10 per cent gelatin and 1.7 cc. in 15 per cent gelatin were now the limits of the jellifying power.

DISCUSSION

Manifestly, in the case of both agar and gelatin, strong acid or alkali in the presence of high temperatures is capable of destroying the jellifying power. Everyone who has made culture

media according to accepted methods, i.e., sterilized them after the adjustment of reaction, has found that an acidity of 2 to 2.5 per cent normal HCl or a pH concentration of approximately 4 to 3.5 is the limit of solidification of agar. Alkalis in related proportions in the presence of heat have been found to exert a similar action on the jellifying power of agar. Fellers (1917), however, found that this range of jellifying power for 2 per cent agar could be extended to 5 per cent normal HCl or 5 per cent KOH if the acid or alkali were added while the agar was boiling hot and it was not subsequently sterilized. These highly acid or alkaline media were furthermore employed by him (1916) in studies on soil flora, since appropriate quantities could be transferred by means of a sterile pipette to sterile Petri dishes. When one permits the media to cool before adding the acid or alkali as was done in our studies, and as is indicated in Fellers' work, the range of solidification may be extended very much farther. The application of the principles involved herein are believed to make it possible both to simplify the making of media and to improve methods for investigation on the influence of hydrogen ion concentration on microörganisms. Reference to two recent papers one by Webb (1919) on the influence of reaction on the germination of fungous spores and the other by Fred and Davenport (1918) on the growth of nitrogen assimilating bacteria, will illustrate the possibilities which may come in similar studies from the use of very acid or very alkaline solid media.

In routine work it will be found to be advantageous to flask and sterilize the media in 200 cc. quantities for the reason that the addition of 1 or 2 drops of strong acid or alkali to this quantity will bring about a change in concentration of about pH 0.2. When acid is added to agar in flasks at 50° to 60°C. it may be thoroughly agitated by whirling, 10 cc. portions may be removed for comparison in reaction with the color standards of Clark and Lubs (1917), and when the usual precautions against contamination are observed the material in the flasks may be kept sterile, while the adjustment to the desired pH concentration is being made. The agar may then, before it has had time to solidify, be poured into sterile test tubes or sterile Petri dishes, where-

upon it is ready for use. The danger of contamination from this procedure, as judged by experience in making several thousand tubes of media, is no greater than when the tubes are sterilized after the media has been placed in them, as is usually done. In summary, this procedure removes the necessity of sterilization after adjustment of reaction, eliminates the chances of change of reaction or of other chemical changes which may be hastened by a rise in temperature, and does not, within a wide range, destroy the jellifying powers of the agar or gelatin.

CONCLUSION

Agar or gelatin media, if cooled before being made acid or alkaline, will jellify at limits far beyond pH concentrations tolerated by microörganisms. They may be manipulated so as to avoid contamination during adjustment of reaction and need not be subsequently sterilized.

REFERENCES

CLARK, W. M., AND LUBS, H. A. 1917 The colorimetric determination of hydrogen ion concentration and its applications in bacteriology. Jour. Bact., **2**, 1–34, 109–136, 191–236, f. 1–8.

FELLERS, C. D. 1916 Some bacteriological studies on agar. Soil Sci., **2**, 255–290.

FELLERS, C. D. 1917 The analysis, purification, and some chemical properties of agar-agar. Jour. Indus. Eng. Chem., **8**, 1128–1132.

FRED, E. B., AND DAVENPORT, AUDREY 1918 Influence of reaction on nitrogen assimilating bacteria. Jour. Agr. Res., **14**, 317–336.

WEBB, R. W. 1919 Germination of the spores of certain fungi in relation to hydrogen ion concentration. Ann. Mo. Bot. Garden, **6**, 201–222.

STUDIES ON AZOTOBACTER CHROOCOCCUM BEIJ.

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I. GENERAL

Introduction

The study of the metabolism of Azotobacter has been generally approached from the standpoint of the rôle of this organism in the nitrogen cycle in Nature, but the fact that the ability to fix free nitrogen is regulated by the presence or absence of combined nitrogen in the medium has not been given serious attention although it is made plain in the works to be cited.

Bejierink and Van Delden (1902) have shown that *Azotobacter chroococcum* possesses the power to transform nitrates directly into ammonia and Lipman (1903), Stoklasa (1908), Stranak (1909) and Heinze (1906) found that small quantities of nitrates "*stimulated*" nitrogen fixation by this organism. If the property of nitrogen fixation were a function of the normal life cycle, it seems strange that, although of vital importance, it should be overcome with such facility. In this connection the data presented by Hills (1918) are most instructive. The accompanying table is a recalculation of the data given by him on pages 200–203 of his contribution, and is chosen as it is the only one given which was obtained by the use of a synthetic medium.

From this compilation we see that in presence of abundant stores of nitric nitrogen Azotobacter does not fix atmospheric nitrogen but assimilates the nitrogen of the nitrates. A close study of the original data shows that in the presence of ammonium nitrate the organism has a preference for the nitrate radicle leaving the ammonium radicle untouched.

The most important feature of the recalculated data, and one that the author apparently overlooked, is the one presented in the last column of table 1. A very appreciable "*loss*" of nitrogen takes place from the cultures, either as free nitrogen or as a volatile nitrogenous compound. It is assumed that the cultures used by Hills were pure.

Greaves (1918) in his recent review of this work seems not to consider this important phase of the metabolism of Azotobacter.

TABLE 1

Nitrogen balances in the cultures of Hills

TYPE OF ORGANISM	TREATMENT OF SOLUTION	TOTAL NITROGEN		NITROGEN UNACCOUNTED FOR, PROBABLY LOST BY BACTERIAL ACTION
		Inoculated	Check	
A	$NaNO_3$	105.20	164.95	59.75
	NH_4NO_3	162.12	206.75	44.63
B	$NaNO_3$	135.60	164.95	29.35
	NH_4NO_3	192.85	206.75	13.90

After this very brief survey of the data in question it may be well to recall a statement which appeared in a previous communication from this laboratory (1915) to the effect that *A. chrococcum* may be a fixer of atmospheric nitrogen only under such conditions as we call "normal," i.e., in absence of fixed nitrogen and a denitrifier when such conditions are changed, i.e., when there is a possibility for it to consume nitrate under naturally normal conditions. In our present study of the literature we have excluded purely agronomic investigations and have considered only studies which have been made under conditions of control such as to give results of fundamental importance.

Historical

A simple study of the relation of the carbon consumed to the nitrogen fixed has only a limited value, and this is especially true when we consider the great variations in the nitrogen fixing power of the same organism under different conditions. The quality of the carbohydrate utilized in the experiment and its

relation to the fixation of nitrogen have received considerable attention but it should be stated nevertheless that the ratio C:N, as often reported in the literature, has no absolute value since the determination of the carbon actually utilized has not been attempted, a complete consumption of all the carbohydrate originally present having been assumed.

Furthermore it must be stated that by following the practice of allowing all the carbohydrate to disappear from a solution the cultures are submitted to a negative phase, one of actual starvation which, as will be seen later vitiates the results of the experiments.

The excellent work of Koch and Seydel (1912) on the influence of the concentration of carbohydrate is typical of the results to be obtained when the concentration of sugar and time of incubation are made elements in an experiment. In fact from their work it is evident that each period of incubation has an optimum concentration of sugar a finding that makes it impossible to draw definite conclusions from data obtained with a given sugar concentration and an arbitrary number of days of incubation.

Stoklasa (1908), using glucose as a source of carbon, concludes that the products of carbohydrate attack by Azotobacter are: ethyl alcohol, formic acid, acetic acid, butyric acid (only once in anaerobic conditions), lactic acid, carbon dioxid and hydrogen. From his data it would appear as if the glucose actually incorporated by the cells during the period of his experiments should be represented by

	grams
Glucose actually consumed	15.8900
Glucose found in byproducts	9.3790
Glucose theoretically in cells	6.5110

But Stoklasa sums up his observations by stating that he cannot account for this quantity of 6.5110 grams of glucose and that probably not all the by-products were determined. A second possible reason for such unaccounted for glucose he assumes to be found in the inaccuracy of the methods for the determination of the by-products (volatile and non-volatile acids).

To reconcile this work with the statements of Omeliansky and Sewerowa (1911) is impossible if we assume both groups of workers to be working with pure cultures of the organism in question. Although statements as to the purity of his cultures were made by Stoklasa, the fact that hydrogen in the free state was found to be generated in appreciable quantities, as well as butyric acid, in some cases, would tend to make this purity questionable.

If now we consider in this connection the work of Krzmieniewsky (1908) we have a series of most interesting data that bring out two very striking features: 1, complete lack of hydrogen production and, 2, striking difference in the CO_2:O_2 ratios resulting from the utilization of glucose and mannitol. One feature of this work which deserves special attention is that the ratio of CO_2 formed to nitrogen fixed is not constant. Expressed in other words there does not exist a constant C:N ratio for Azotobacter. The nitrogen fixation from the atmosphere is such that it cannot be considered as a normal and essential function, necessary to the cell economy of the organism, but rather as an incidental or secondary factor in the cell metabolism. As an addendum to the main bulk of his work Krzmieniewsky mentions a series of experiments which fail to corroborate the findings of Stoklasa, with regard to the formation of by-products, and cast thereby additional doubt on the purity of the cultures of the latter investigator. That this difference in the metabolism (production of organic acids and alcohols) may not be due to differences in the organisms studied or, necessarily, to impurities is shown by some of the data presented by Mazé (1902).

That an organism such as *Eurotiopsis gayonii* is capable of changing its physiology and the nature of the by-products of its life activities (under anaerobic conditions performing only the process of intramolecular respiration) would tend to convince us that Stoklasa might not have been working with impure cultures but may rather have been growing his cultures under conditions of anaerobiosis. That such a process of anaerobiosis is possible in Azotobacter may be postulated when we consider the work of Mazé on the assimilation of glycerol, lactic acid and aldehyde by Eurotiopsis.

Nevertheless, although lactic acid and alcohol could be assumed as by-products of an abnormal physiology of Azotobacter, the production of hydrogen and butyric acid suggests a contaminating form.[1]

The fact that Azotobacter gave a ratio CO_2:O_2 of about unity in Krzmieniewsky's work does not in itself indicate that all the carbohydrate consumed is utilized in a process of combustion. In fact, it is unfortunate that with such a careful study of the gaseous exchange in their cultures these authors were not in a position to present data showing the actual disappearance of the carbon source from the solutions.

If we consider the carbon balances in the mathematical terms introduced by Duclaux (1900) we obtain the following equation.

$$S = m.L + n.l.t \qquad (1)$$

where S is the quantity of sugar consumed, L the weight of the cells at the end of the incubation period, l that quantity of cellular substance that constitutes the average throughout the period of experiment, m the quantity of sugar actually to be found in the mass of one unit of cellular substance, n that quantity of sugar necessary for the maintenance of one unit of cellular substance during the unit of time t.

Omeliansky and Sieber after a study of the composition of the cells of Azotobacter (1913) grown on agar concluded that cells of Azotobacter contained a relatively small quantity of protein (about 13 per cent), their greater mass being made up of nitrogen-free substances. It is possible that the heavy "schlimeschicht" that surrounds the cells during the early stages of development should not be forgotten in this connection as also the peculiar granulations to be found in the cells of this organism (Bonazzi, 1915) and which play a rôle in their life cycle that is not as yet completely understood. It is possible that such structures are largely made up of that sugar carbon which has been denominated m in the above equation. But as will be

[1] Krzmieniewsky admits of the possibility that the organism which Stoklasa was studying was not Azotobacter.

seen later these same structures are utilized in future life and should therefore be considered again in the calculation of n, that quantity of sugar which goes to the maintenance of the cell.[2]

The above analysis of the literature leads us to the following considerations: a) the C:N ratio of Azotobacter is an inconstant value, b) the metabolism of this organism is not as yet well understood, c) the nitrogen fixing capacity of this organism seems to be a function of secondary importance in the cell economy.

II. EXPERIMENTAL

Carbon relations

CO_2:O_2 ratio and sugar consumption

Experiment 44. A large flat-bottomed Fernbach flask was fitted with the attachment shown in figure 1.

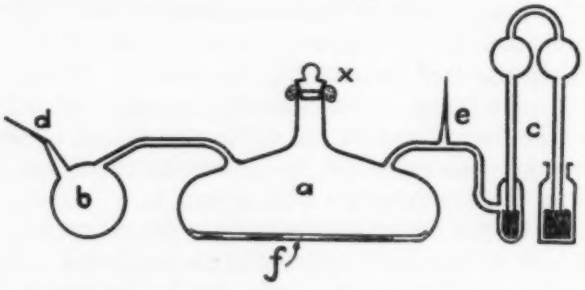

FIG. 1. *a*, culture flask; *b*, *d*, *e*, gas collecting chain; *c*, mercury valve; *f*, culture solution; *x*, paraffin and plaster of paris seal fitted over the ground glass stoppers to avoid possible gas leaks.

The total volume of the apparatus was 3290 cc. and since 100 cc. of solution were used, the total volume of air over the solution was 3190 cc. The solution used was of the following composition:

Deep well water	500.00 cc.
K_2HPO_4	0.10 gram
Glucose	10.00 grams

[2] Krainsky obtains curves on the CO_2 production of cultures of Azotobacter but unfortunately gives no data on the actual sugar consumption.

One hundred cubic centimeters of this solution were placed in the flask together with 0.50 gram of precipitated $CaCO_3$. After sterilization and inoculation with a pure culture of *Azotobacter chroococcum* the whole apparatus was sealed and incubated at 25°C. for forty-eight days. A gas analysis at the start of the incubation period and at the end gave the results shown in table 2.

TABLE 2

Gas changes in culture of Azotobacter chroococcum

GAS	COMPOSITION OF AIR				TOTAL GAS BALANCES
	At start		At the end		
	Per cent	Actual	Per cent	Actual	
		cc.		cc.	cc.
CO_2	0.00	0.00	9.70	309.40	+309.4
O_2	20.30	647.60	11.33	361.40	−286.2
H_2	0.00	0.00	0.00	0.00	0.0

Calculating the ratio CO_2:O_2 we obtain the value 1.08 which closely approximates the value 1.02 obtained by Krzmieniewsky in his work with glucose. But under such high partial pressure of CO_2 the solution in the flask can contain appreciable quantities of this gas in solution. In fact by titration of the solution with Na_2CO_3 and H_2SO_4 it was found to contain 50.70 mgm. of carbon dioxid. Gravimetrically then, the quantity of CO_2 in the air would be 607.80 mgm., and that in solution 50.70 mgm., giving a total quantity of 658.50 mgm. of carbon dioxid in the system. The oxygen consumption was of 286.20 cc. corresponding to 408.80 mgm. of O_2. A sugar analysis showed the following changes in the solution:

Uninoculated	1840.0 mgm. $C_6H_{12}O_6$
Inoculated	41.2 mgm. $C_6H_{12}O_6$
Total sugar consumed	1798.8 mgm.

Assuming the oxygen to be utilized in the combustion of the sugar according to the following equation (equation 2) which

represents only the final changes, we are in a position to estimate the quantity of CO_2 which should have been formed in the process (table 3).

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6\ CO_2 + 6\ H_2O \qquad (2)$$

The final result of this experiment is therefore to show that more carbon dioxid is formed and more sugar consumed than can be accounted for by the amount of oxygen consumed.

The solution in the particular flask was the seat of vigorous development, beginning with the formation of a ring at the point of air-glass-solution contact, slowly clouding the whole solution and later sinking to the bottom of the flask in the form of a heavy deposit exhibiting the gray-brown pigmentation characteristic of Azotobacter.

TABLE 3

Gas balances in cultures of Azotobacter

	$C_6H_{12}O_6$ CONSUMED	O_2 UTILIZED	CO_2 FORMED
	mgm.	*mgm.*	*mgm.*
Actual	1798.8	408.8	658.5
Calculated	383.2	408.8	562.1
Differences	+1415.6	0.0	+96.4

The quantity of sugar unaccounted for may well have been found in the cell body and secretions had an effort been made to account for the whole. Since this was not done we are only justified in assuming such a possibility. That some secondary actions do take place in a culture of Azotobacter is shown by the presence of 96.4 mgm. CO_2 in excess of the theoretical amount. We may safely assume at present that this quantity of carbon dioxid is derived from a process of intramolecular respiration. A proof of this may be found in a later part of this paper, under the heading of Autophagy. This assumption is also in agreement with the equation of Duclaux for aerobic organisms, and is substantiated by the following experiment.

Experiment 46. A large Fernbach flask fitted with the attachments shown in figure 2, received 100 cc. of a solution of the composition shown below:

Deep well water	250.00 cc.
K_2HPO_4	0.05 gram
Glucose	5.00 grams
$Ca(NO_3)_2 + 4H_2O$	0.316 gram

Calcium carbonate was added in the quantity of 0.5 gram. The total volume of the apparatus was of 2666 cc. and the addition of the solution left 2566 cc. of air space. After sterilization and inoculation with a pure culture of *Azotobacter chroococcum* the apparatus was incubated at about 25°C. for fifty-two days, after which period a gas analysis was made. Comparing the results obtained at the end to the values at the start of the experiment we may calculate the gas exchanges in the culture as shown in table 4.

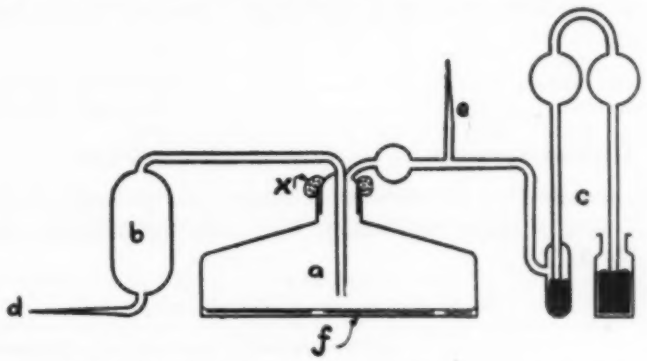

FIG. 2. *a*, culture flask; *b*, *d*, *e*, gas collecting chain; *c*, mercury valve; *x*, paraffin and plaster of paris seals over ground glass joints.

TABLE 4

Gas changes in culture of Azotobacter chroococcum

GAS	COMPOSITION OF AIR				CHANGES IN TOTAL GAS
	Before		After		
	Per cent	Actual	Per cent	Actual	
		cc.		cc.	cc.
CO_2	0.13	3.33	20.70	531.20	+527.87
O_2	20.21	518.50	1.02	26.17	−492.33
H_2					

Calculating the $CO_2:O_2$ ratio we obtain the value 1.07. Here again a very close approach is seen to the value obtained by Krzmieniewsky when glucose was used as a source of carbon. Again here as in the previous experiment the carbon dioxid dissolved in solution was determined and found to be 44.36 mgm. and the quantity of this gas in the atmosphere 1037.0 mgm. The total weight of the gas formed then would be 1081.4 mgm. while a total quantity of 703.3 mgm. of oxygen was consumed. A determination of the sugar concerned in the action follows:

Uninoculated	1701.0 mgm. $C_6H_{12}O_6$
Inoculated	572.8 mgm. $C_6H_{12}O_6$
Total sugar consumed	1128.2 mgm.

If we assume that the consumed oxygen was utilized wholly in the process of sugar combustion we obtain the balances shown in table 5.

TABLE 5

Gas balances in cultures of Azotobacter

	$C_6H_{12}O_6$ CONSUMED	O_2 UTILIZED	CO_2 FORMED
	mgm.	mgm.	mgm.
Actual	1128.2	703.3	1081.4
Calculated	659.4	703.3	967.2
Differences	+468.8	0.00	+114.2

Here again we see indications of an intramolecular respiration taking place with a consumption of sugar above the theoretical.[3]

Comparing now the data from the two experiments mentioned above we have table 6.

It seems evident therefore that the cells of Azotobacter besides retaining abundant stores of the carbohydrate with which they

[3] A third experiment of the series, in which the $CO_2:O_2$ ratio has been determined, gave a value of 1.09 for the respiratory exchange, a value in very close agreement to the others here reported. A summary of the $CO_2:O_2$ ratios follows:

Experiment 44	1.08
Experiment 46	1.07
Experiment 78	1.09
Value of Krzmieniewsky	1.02

are placed in contact secrete or produce in the surrounding medium compounds, the nature of which has not been determined. Before attempting to study these compounds we shall enter into a closer analysis of the above experiments. Expressing the quantity of unaccounted for sugar in terms of percentage of the amount of sugar actually disappeared from solution we obtain table 7.

TABLE 6

Summary of tables 3 and 5; values obtained in excess of theoretical

TREATMENT	$C_6H_{12}O_6$	CO_2
	mgm.	mgm.
None	+1415.6	+96.4
Nitrate	+468.8	+114.2

TABLE 7

Sugar unaccounted for as per cent of the sugar which disappeared

TREATMENT	VALUE
None	78.71
Nitrate	41.56

It is obvious that no relation whatever exists between the sugar unaccounted for and the CO_2 formed in excess of the theoretical, and from table 7 we gather that the addition of nitrogen in the form of Ca $(NO_3)_2$ has induced a profound change in the physiology of Azotobacter. That the nitrogen is actually consumed during the process of growth could be assumed from the published works of other authors, but to obtain more specific results we may summarize the data obtained on the above mentioned cultures (table 8).

The transformation of nearly 10 mgm. of nitric nitrogen into organic nitrogen (which was probably in part organized) is directly connected with the carbohydrate consumption and with the stores of carbon in the cell body and by-products. If such soluble and insoluble material were actually formed it should be possible, by stopping fermentation in its early stages or by an oxygen hunger, to obtain a carbon balance showing a greater

disappearance of sugar than can be accounted for by the actual oxygen consumption; and the carbon dioxid production should be correspondingly diminished. That this is what actually happens is shown by the following experiment.

TABLE 8

Nitrogen balances of Azotobacter

	CULTURE							
	No nitrate				Nitrate			
	NH_3	N_2O_3	Organic	Total	NH_3	N_2O_3	Organic	Total
	mgm. N	*mgm. N*	*mgm. N*	*mgm. N*	*mgm. N*	*mgm. N*	*mgm. N*	*mgm. N*
At start				0.69		17.14	0.88	18.02
At end				3.86		7.28	10.65	17.93
Balances				+3.17		−9.86	+9.77	−0.09

Experiment 79. A number of heavy walled Erlenmeyer flasks of 300 cc. capacity received 50 cc. of a solution of the following composition:

Deep well water	500.00 cc.
K_2HPO_4	0.25 gram
NaCl	0.25 gram
$FeSO_4 + 7H_2O$	0.025 gram
Glucose	5.00 grams

Another set of flasks received 50 cc. of this same solution to which had been added 0.115 per cent of KNO_3. The flasks were all fitted as is shown in the accompanying figure 3 with manometer tubes.

They had all received in addition to the solution 0.25 gram of precipitated $CaCO_3$. One flask in each series (nos. 1 and 5) was left uninoculated to serve as control while the others were inoculated with a pure culture of *Azotobacter chroococcum*. After varying periods of incubation at 27°C. the gas was pumped out of the flasks by means of a mercury pump until the solution boiled at about 30°C.; then boiling was continued for a few minutes, and the gas mixture thus obtained was analyzed. The

solution was then used for the determination of the residual sugar. The results are presented in tables 9 and 10.

Expressing these values in terms of cubic centimeters of gas in the total volume of the flasks at 0°C. and 760 mm. we have the result shown in table 10.

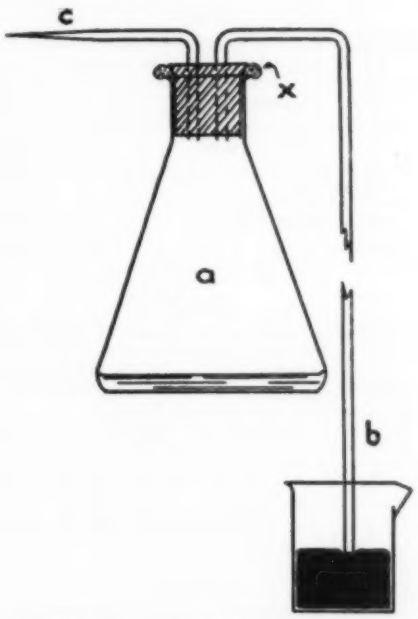

FIG. 3. *a*, culture flask; *b*, manometer tube (Hg rose to atmospheric pressure when evacuated); *c*, evacuation tube and gas collector connection; *x*, paraffin coating on rubber stopper.

If now we consider all the oxygen disappeared to be used in a process of combustion such as is expressed in equation 2 on page 338, we have the data given in table 11.

In the above data we find confirmation of the hypothesis expressed above. Azotobacter utilizes the sugar first in the building up of its cell substance and the preparation of non-reducing substances; and slowly digests these "stores" of carbonaceous material in the process of later development in the

presence of oxygen. It is also reasonable to assume that the energy resulting from this oxidation is utilized in further growth. If this were actually the case, it should be possible to follow these various steps and the coincident disappearance of sugar at close intervals of time, in a solution undergoing active fermentation.

TABLE 9

Results of gas analyses of cultures of Azotobacter under oxygen starvation expressed in percentage of the gas mixture

GAS	NO NITRATE				WITH NITRATE			
	Number of flask				Number of flask			
	1	2	3	4	5	6	7	8
CO_2	0.00	17.51	17.64		0.00	17.96	19.87	
O_2	20.32	0.49	1.94		20.27	0.80	0.97	

TABLE 10

Gas and sugar changes in cultures of Azotobacter

TREATMENT	NUMBER	DAYS OF INCUBATION	OXYGEN CONSUMED		CARBON DIOXID FORMED		SUGAR CONSUMED	$\frac{CO_2}{O_2}$
			cc.	mgm.	cc.	mgm.	mgm.	
None	2	3	45.84	65.49	40.48	79.51	86.20	0.88
	3	4	42.93	61.33	42.16	82.82	76.80	0.98
Nitrate	6	3	45.79	65.41	42.24	82.97	70.00	0.92
	7	4	47.67	68.10	49.08	96.41	72.80	1.03

TABLE 11

Gas balances in cultures of Azotobacter chroococcum

TREATMENT	NUMBER	DAYS OF INCUBATION	$C_6H_{12}O_6$ CONSUMED ABOVE THEORY	CO_2 FORMED ABOVE OR BELOW THEORY
None	2	3	+24.80	−10.49
	3	4	+19.30	+1.51
Nitrate	6	3	+8.68	−6.97
	7	4	+8.55	+2.77

This method of study has been followed by Allen (1920) with the results diagrammatically shown in the figure 4.

The sharp fall of the sugar contents during the period *O-X'* from the quantity *Y* to *Y'* shows without doubt that the sugar

is worked over by the cells during the early stages of cultivation and is then slowly utilized by the cells in their later development, during that period when the sugar curve runs about parallel to the asymptote (to the axis X).

In another experiment the following data were obtained, corroborating the above statement.

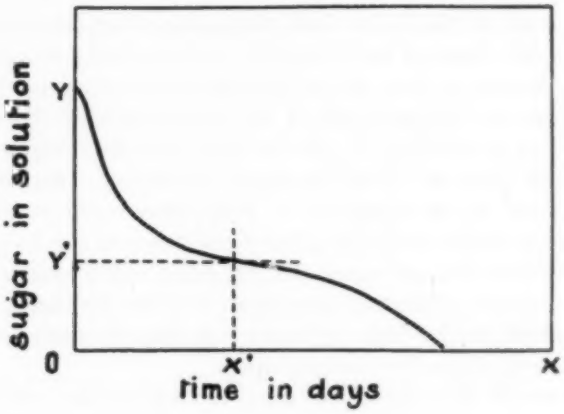

FIG. 4

Experiment 51. Sugar consumption in large petri dishes of 20 cm. diameter:

	FIRST 5 DAYS	NEXT 3 DAYS	NEXT 5 DAYS
	mgm.	mgm.	mgm.
Sugar consumed	316.7	160.0	0.0
Consumed per day during periods	63.0	53.0	0.0

Keeping in mind the fact that there are very few cells active during the first five days, the number in fact being negligible during the very first day or two, we can see that the first value is much more than 63 mgm. of sugar consumed per day.[4]

[4] Here it may be well to keep in mind the mathematical interpretation of bacterial growth presented by Duclaux and discussed by him in volumes 1 and 2. This interpretation was later included by Rahn in his treatment of the fermentive capacity of a single cell. Mich. Res. Bul. 10.

It is unnecessary to present more data on this point since it would all corroborate the above statements without adding new facts of importance. It is nevertheless important to obtain a crucial test of the actual storing and utilization of the stores of the sugar carbon; such evidence follows.

Autophagy of Azotobacter

That some of the sugar which disappears from solution during the first few days or hours of growth is actually stored in the cells is obvious in view of the fact that the carbon of the sugar contributes to the synthesis of the compounds of the cell substance, but in addition as will be seen, Azotobacter presents an interesting case of what Duclaux designates with the term "phénomène de vie continuée." This phase is one in which the organism is really living on its own reserves and the by-products of its previous life activities, just as yeast will continue to live in a fermented mixture at the expense of the glycogen, glycerol and succinic acid which it formed during its early stages of development and active fermentation.

Experiment 19. Pure cultures of *Azotobacter chroococcum* were made on Ashby's mannitol-washed-agar plates and allowed to incubate for twenty-four hours.

The growths thus obtained were emulsified in 0.75 per cent NaCl solution and aseptically placed in sterile test tubes. Slides of this bacterial suspension were prepared immediately and after forty-two and one hundred and fourteen hours' standing in the incubator. They were stained by means of the Giemsa solution which stains well the peculiar granulations studied in a previous communication (Bonazzi, 1915). Examining fifty microscopic fields at random on each of the slides so prepared, and counting the number of cells containing granules (granulated), those free of granules and those in which the granules have partially disappeared (transitional) the following data were obtained.

The cells here classed as transitional are those in which the granules had nearly disappeared or were greatly diminished in size and could well be classed among the ungranulated. If this were done the following table would be obtained.

From these data it seems evident that the stores which were accumulated by the cells during the first twenty-four hours of development on a complete medium were subsequently attacked when no more sugar was at their disposal. Nevertheless it must be stated that the granules may not be the only stores of the Azotobacter cell, and it is very probable that the heavy gelatinous capsule described by various authors is also a storing as well as a protecting organ.

TABLE 12

Autophagy of Azotobacter

HOURS OF INCUBATION	PER CENT OF TOTAL NUMBER OF CELLS AS		
	Granulated	Transitional	Free of granulations
0	84.62	6.15	9.23
42	8.52	19.28	72.20
114	9.02	28.18	62.80

TABLE 13

Autophagy of Azotobacter

HOURS OF INCUBATION	NORMAL CELLS	AUTOPHAGED CELLS
0	84.62	15.38
42	8.52	91.48
114	9.02	90.98

Sugar: cells ratio

Among the assimilation products of Azotobacter we should also consider such compounds as play only a transitory rôle in the cell metabolism and are later secreted in the surrounding medium. Stoklasa believes these to be ethyl alcohol, lactic, acetic and butyric acids, but we have seen that we should question his results since we have reason to accept Krzmieniewsky's data and Omeliansky's statements with regard to these formations. That no fixed acids are present has not as yet been shown with certainty (although Omeliansky points to their absence) while no volatile acids have been found in an Azotobacter culture by Krzmieniewsky. Repeated trials made in this laboratory in the hope of finding whether the unaccounted for sugar could be

found in the form of volatile acids, failed to reveal their presence when the cultures were distilled in presence of tartaric acid. Nevertheless, whatever these compounds are they do not seem capable of stopping the development of Azotobacter as is shown in the following experiment.

Experiment 71. A series of flasks containing each 25 cc. of a glucose solution, $Ca(NO_3)_2$ and $CaCO_3$ was inoculated with a pure culture of *Azotobacter chroococcum* and incubated at 30°C. One flask was left uninoculated as a control. After the necessary period of incubation had elapsed the solutions were acidified until complete solution of the carbonate, allowed to stand in this acidified condition for a short time and then filtered with suction, through a crucible filter prepared according to the accompanying illustration (fig. 5).

FIG. 5. *a*, perforated glazed crucible; *b*, glass wool; *c*, washed, digested, ignited quartz sand; *d*, asbestos; *e*, packed and burnished platinum sponge.

The solutions passing through the filter were perfectly clear showing that the cells were completely retained by the filter. Careful washing with water, in small quantities at a time, insured complete removal of the retained sugar. By this procedure it was possible to separate the cells from the surrounding medium so as to form a conception of the ratio $S:c$ where S is the sugar consumed and c the weight of the cells produced, reckoned in terms of cellular substance dried at 110°C. in vacuum, over P_2O_5.

One of the above mentioned cultures, no. 5, was not filtered but received instead the addition of 2 cc. of a 12.5 per cent glucose solution under aseptic conditions; allowed to incubate for a longer period of time it was then subjected to the same

treatment as the others. The results of this experiment are tabulated in table 14.

TABLE 14

Effect on the growth of Azotobacter chroococcum of its own by-products

NUMBER OF CULTURE	$C_6H_{12}O_6$ FOUND				DAYS OF INCUBATION	DRY MATTER		S:c
	At start	At end	Consumed	Consumed in second period		Actual	Increase in second period	IN EACH PERIOD
	mgm.	mgm.	mgm.	mgm.				
1	162.0	162.0	0.0		0			
2	162.0	7.1	154.9		30	18.2		8.51
3	162.0	9.7	152.3		30	Lost		
4*	162+214.7							
	=376.7	376.7	0.0					
5	376.7	6.2	370.5	216.9	38	27.3	9.1	23.84

* Total quantity of sugar at the beginning of second period.

Addition of carbohydrate to a culture that has come to a standstill will result in a new utilization of the carbon source with further growth. The first compounds resulting from the first utilization of the sugar had probably all been utilized during the first thirty days of incubation and only such by-products as the cells could not well utilize were to be found when the culture received the fresh supply of sugar; that these were not inhibitive is shown by the utilization of the sugar in the second period as well as by the additional growth. Their nature will be studied elsewhere, and at present it is sufficient to state that they are formed through the cell activity.

Ferment powers of Azotobacter

From table 14 we may see that the conception S:c is erroneous with regard to the data for culture 5. In fact while it is true that there was a renewal of growth in the second period with a corresponding increase in dry matter, we are not justified in considering that it was only the 9.1 mgm. additional growth that consumed the additional sugar, but we should deem it possible that the 18.2 mgm. of the cells already present in the culture used the new source of carbon for their maintenance. In other

words, referring to equation *1* set forth on page 335 of this memoir we may well express our hypothesis as follows: during the first period of incubation there was a balance between the two functions represented by the equation, while in the second period of incubation the addition of sugar disturbed the balance in such a way that although the first member of the second term was active on 9.1 mgm. of new cellular substance, the second member of this term was active on a total of 9.1 + 18.3 = 27.4 mgm. This leads us to the conclusion that the conception of "*ferment power*" in an organism such as Azotobacter should be carefully studied. The following series of experiments was therefore designed to study this phase.

Experiment 69. Some 250 cc. Erlenmeyer flasks received 50 cc. of the following solution together with 0.5 gram of precipitated calcium carbonate.

Deep well water	500.00 cc.
K_2HPO_4	0.25 gram
NaCl	0.25 gram
$FeSO_4 + 7H_2O$	0.02 gram
Glucose	5.00 grams
$Ca(NO_3)_2 + 4H_2O$	0.632 gram

TABLE 15

Sugar consumed per unit of dry matter in cultures of Azotobacter

NUMBER OF CULTURE	DAYS OF INCUBATION	SUGAR CONSUMED	DRY MATTER OF CELLS AND SLIME	S:c	
				Actual	By periods
		mgm.	mgm.		
5	0	0	0.0	0.00	0.00
6	3	65	Lost		
7	5	109.8	12	9.15	9.15
8	23	411.5	62	6.63	6.03

After inoculation with equal quantities of a pure culture of *Azotobacter chroococcum* the flasks were incubated at 30°C. for varying periods of time. One of the flasks was left uninoculated to serve as control. The dry matter in the cultures was determined by acidifying, filtering in the manner referred to above, washing and drying at 110°C. in vacuum over P_2O_5, while the sugar was determined in the filtrate. The results are set forth in table 15.

Before entering into a discussion of the above data other experiments will be related.

Experiment 81. Twenty-five cubic centimeters of the solution mentioned in the previous experiment were placed into 1500 cc. Erlenmeyer flasks together with 0.25 gram of $CaCO_3$ and sterilized, inoculated with equal and very small quantities of

TABLE 16

Sugar consumed per unit of dry matter in cultures of Azotobacter—experiment 81

NUMBER OF CULTURE	DAYS OF INCUBATION	SUGAR CONSUMED	DRY MATTER OF CELLS AND SLIME	S:c	
				Actual	By periods
		mgm.	mgm.		
6	0	0.0	0.0	0.0	0.0
7	1	1.4	0.0		
8	2	4.4	0.0*		
9	4	69.2	6.3	10.9	10.3
10	9	184.8	24.8	7.4	6.2

* Pronounced opalescence of solution too little to be weighed.

TABLE 17

"Ferment power" of Azotobacter chroococcum

DAYS OF INCUBATION	FERMENT POWER S:c.t*
30	0.28
23	0.29
9	0.82
5	1.83
4	2.74†

* S:c.t, where S sugar consumed, c cellular dry matter and t time in days.

† This value is in reality calculated differently from the others. But since on the second day of incubation no appreciable amount of cell substance was formed (experiment 81, no. 8) we should be justified in calculating this value just as the others, i.e., on the base of a two days' growth; thus a "ferment power" = 5.45.

Azotobacter chroococcum and incubated at 30°C. for varying periods of time. The same analytical technique was used in the analysis of these as in the cultures of the previous experiment.

Comparing now the data presented in tables 14, 15 and 16 we see that the culture incubated thirty days gave a S:c ratio of

8.51 while the culture incubated twenty-three days gave a value 6.63—of approximately equal magnitude. From these data we can draw a very interesting set of figures if we consider the actual "*ferment power*" per day. The term "*ferment power*"

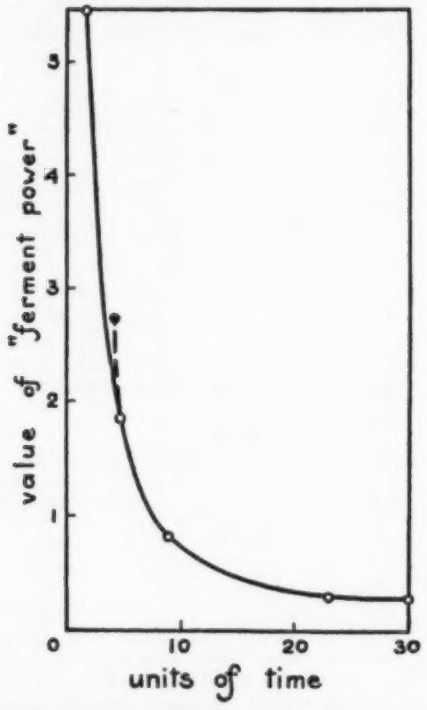

FIG. 6. ● = value of 4 day period on 4 day basis; ○ = value of 4 day period on 2 day basis (see text).

as used by Pasteur and modified by Duclaux to include the time element gives valuable indications as to the physiology of Azotobacter (see table 17 and fig. 6).

The term "ferment power" is here used to designate that quantity of sugar consumed by the unit of dry cell substance in the unit of time under the conditions of the experiments. This concept leads us to the conclusion that during the early stages of

development in a culture there is a greater transformation of the crude food substances than during the later stages, a conclusion that is corroborated by the opinion expressed in the previous pages.

We see thus: 1, that the term "*ferment power*" should not be considered as a function, constant throughout the life cycle of Azotobacter and, 2, that an organism such as this is capable of utilizing the carbohydrate of a culture in a process of "storage" or transformation without a corresponding cellular development.

General considerations on carbon relations

In the preceding pages we have assumed that that quantity of sugar carbon as such which disappears from the solution is to be found in the cells and their by-products. That it is not to be found in the cells themselves is shown by the high coefficient of "*ferment power*" in a young culture of the organism in question, since this value is based on that quantity of sugar that is "consumed" by the unit of cell substance in the unit of time.

Objection could be raised to the conception "unit of cell substance" only on the basis of numbers of active cells since the law of multiplication, when all factors remain equal during the duration of the experiment, makes the number of cells found at the end of an incubation period equal to the number that has been active throughout this period, provided the number of cells at the beginning is considered as unity.[5]

Nevertheless the final weight of cellular dry matter in a culture represents the algebraic summation of the two opposite phenomena of anabolism and catabolism, a value related not only to the size of the inoculum itself, but also to the activity of the organism concerned.

In other words, it furnishes an index both of the "growth capacity" of the organism and its ability to build living bacterial substance, as well as of its actual capacity as a ferment.

[5] Expressed in terms of equation: $N = n - 1$ where N is the number of units at the end and n is the number of units actually active during the whole period of incubation, each unit of the same number of cells as the original inoculum which has multiplied in geometrical proportion on incubation.

We thus see that the sugar lost from a culture in the early stages of development passes through the cells in large quantities and is transformed into compounds which do not form integral part of the cells themselves but are dissolved in the medium. The curves of CO_2 production given by Krainsky (1908, 1910) might indicate the close relation between this function and growth, which is pointed to by the values of the ferment power here obtained.

This conception constitutes the conclusion to be drawn from the discussion and closes, temporarily, the chapter on carbon relations of Azotobacter. That it appears contrary to the conclusions of Krzmieniewsky and Omeliansky and Prazmowsky is evident, but it should be remembered that their search for soluble by-products was performed ten days after the start of the experiments,—probably when they had been already utilized. This is significant in view of the fact that we failed to find volatile acids in our cultures in conformity with the findings of the above mentioned investigators.

Nitrogen relations—Attack on nitrates by Azotobacter

The organism with which these investigations were undertaken was an organism from the Wooster soils that when grown in twenty-five cubic centimeters of Ashby solution (1 per cent mannitol) in 150 cc. Erlenmeyer flasks and incubated for fifty-nine days at a temperature of 28°C. possessed a low nitrogen fixing capacity.

Experiment 15.

B-1 check uninoculated (mgm. N_2 found)	0.65
B-3 inoculated culture (mgm. N_2 found)	2.78
Nitrogen fixed (mgm.)	2.13

Thus fixation calculated to the basis of 1 liter of solution would give 85.2 mgm. fixed nitrogen; a quantity that represents a fixation of 8.5 mgm. of nitrogen per gram of mannitol if we assume all the mannitol to be utilized during the experiment.

When this same organism was grown in 100 cc. of a 2 per cent mannitol solution in large Fernbach flasks it fixed the following quantities of nitrogen:

Experiment 29–31.

	Incubation in days	
	8	*17*
Check uninoculated (mgm. N_2 found)	0.66	0.97
Inoculated (mgm. N_2 found)	3.61	7.47
Nitrogen fixed (mgm.)	2.95	6.50

Incubation was done on a klinostat where the solution was kept in continual movement and the layer of solution was never above 0.5 cm. deep. (Bonazzi, 1919.) The organism proved itself to be a nitrogen fixer in the ordinary sense of the term, as it was found capable of utilizing the atmospheric nitrogen. The mannitol in these cultures was not all consumed and it was impossible to study the nitrogen fixation per gram of mannitol consumed.

From the work of the various investigators quoted in the first part of this memoir, it can be seen that *Azotobacter chroococcum* may utilize nitrates when grown in their presence. From the data reproduced as table 1 of this contribution Hills draws the following conclusion: "In regard to the fixation of nitrogen by these strains of Azotobacter it was found that nitrogen was assimilated both in presence and absence of nitrates. It seems evident that sodium and ammonium nitrate in the amounts studied did not prevent the fixation of the atmospheric nitrogen. In fact the presence of these salts seemed to stimulate the process." Again emphasis is placed on this interpretation when the author states: "However in contrast to the work of Stoklasa, both strains of Azotobacter assimilated more atmospheric nitrogen in presence than in absence of these salts."

From table 1 of the present memoir, where Hills' data are recalculated we see that such conclusions are wholly unjustified. Especially is this true when we consider that the analytical method used for the determination of nitrates includes the nitrite nitrogen as well. This speaks against the assumption that "the reduction of nitrates by Azotobacter takes place with

the formation of nitrites as is shown in table 14." By referring to table 14 of this author we find that 18.9 mgm. of nitrate nitrogen were lost with a resulting "slight" reaction for nitrites in the solution. A concentration of 18.9 mgm. of nitrites in 100 cc. of solution (for that matter even much less than this) gives more than a "slight" reaction with such a sensitive reagent as the Tromsdorff solution, and it is more than questionable if the totality of the nitrate lost is to be found in the form of nitrites. In addition the analytical data point to the incorrectness of this view.

It is a common experience to see a good development of Azotobacter in cultures containing nitrates while poor development takes place in cultures containing no nitrate. For the sake of argument we may assume that the nitrate acts solely in virtue of the stimulation it is supposed to exercise on the nitrogen fixing power of the organism. This increase in the nitrogen fixing power, if present at all, is relatively small and amounts to only 200 to 500 per cent of the original fixation; a stimulation that, when we consider the small original fixation, is relatively small. Although actual data are wanting, we may assume this stimulation to amount to 500 per cent (see the data of Hills on the influence of nitrates on the nitrogen fixation in sterilized soils). A relative increase of 500 per cent in nitrogen fixation brought about by an increase in the number of active cells of 3150 per cent represents an inconceivable stimulation in the fixation of nitrogen, every cell actually fixing less nitrogen in presence than in absence of the fixed nitrogen. It must furthermore be admitted that such a nitrate addition stimulates growth in a different measure than it does nitrogen fixation. Here again we obtain proof of the fact that the nitrogen fixing capacity of the cell is not intimately connected with the function of growth and reproduction.

If the stimulation hypothesis is to be accepted how are we to consider such a difference in these two powers? We are in reality more justified in considering the nitrates as stimulating (or better still aiding) growth in the first place. Basing our working hypothesis on the physiology of the organism we see

that the nitrates increase growth of the cells and their multiplication with a corresponding increase in sugar consumption, and in this process the nitrates disappear to be later found in the organic form, and only after such a phenomenon has taken place does the atmospheric nitrogen fixation really become active.

Experiment 27. Fifty cubic centimeter portions of a solution of the following composition were placed into 500 cc. Kjeldahl flasks together with 0.1 gram $CaCO_3$.

Mannitol	20.0 grams
$MgSO_4 + 7H_2O$	0.408 gram
NaCl	0.200 gram
$CaSO_4 + 7H_2O$	0.127 gram
K_2HPO_4	0.200 gram
Tap water	1000.00 cc.

TABLE 18

Nitrogen fixation by Azotobacter in presence and absence of nitrates

TREATMENT	NUMBER OF CULTURE	NITROGEN AS				AVERAGE OF TOTAL NITROGEN	NITROGEN FIXED
		NH_3	N_2O_3	Organic	Total		
		mgm.	mgm.	mgm.	mgm.	mgm.	mgm.
None:							
Check	1			0		0	
Check	2			0		0	
Inoculated	3			*			
Inoculated	4			0.81	0.81	0.81	+0.81
Nitrate:							
Check	5	0.25	5.43	*			
Check	6	0.21	5.30	1.76	7.35	7.35	
Inoculated	7	0.19	4.37	1.98	6.54		
Inoculated	8	0.18	3.79	1.98	5.96		
Inoculated	9	0.17	4.50	1.89	6.56		
Inoculated	10	0.13	4.54	1.96	6.62		
Inoculated	11	0.15	4.10	*		6.38	−0.97

* Determination lost.

Eleven flasks were prepared and numbered successively from 1 to 11. They were arranged as follows: Nos. 1–4 inclusive received in addition to the above solution 5 cc. of water and flasks 5–11 inclusive received 5 cc. of a 0.843 per cent solution of $Ca(NO_3)_2 + 4H_2O$. All the flasks except nos. 1, 2, 5, 6 were

inoculated with a pure culture of *Azotobacter chroococcum*. After an incubation of nineteen days at 30°C. the cultures were analyzed and found to give the values summarized in table 18. Reference to the cultural notes shows that by far the better growth was found in the nitrate cultures during the whole period of incubation.

An attack on the nitrate is here evident, without a quantitative corresponding increase in the organic nitrogen. Furthermore a certain amount of nitrogen (that quantity which failed to be organized) is actually lost from solution. Although the data are not quantitatively conclusive, their qualitative significance is paramount. They establish an actual loss from the solution in direct corroboration of the results of Hills. Since the depth of the solution layer may be responsible for the low nitrogen changes obtained, trials were made with extensive surfaces of exposure.

Experiment 32. Four large Fernbach flasks received 100 cc. of Ashby's solution together with 0.5 gram $CaCO_3$; two of these

TABLE 19

Nitrogen fixed or lost by Azotobacter in absence or presence of nitrates

TREATMENT	NUMBER OF CULTURE	NITROGEN AS			GAIN OR LOSS
		Organic and ammonia	Nitrous and nitric	Total	
		mgm.	mgm.	mgm.	mgm.
None:					
Control	2	0.66		0.66	
Inoculated	5	3.61		3.61	+2.95
Nitrate:					
Control	4	0.88	16.81	17.69	
Inoculated	6	15.84	1.38	17.22	−0.47

flasks received 5 cc. of water while the other two received 5 cc. of a 2.532 per cent solution of $Ca(NO_3)_2 + 4H_2O$. The flasks intended for inoculation were sterilized and all the flasks then received as infecting material equal amounts of a suspension of *Azotobacter chroococcum*. After this the controls were sterilized at the same temperature and pressure as the others.

After eight days' incubation at 25°C. the cultures were analyzed and found to give the values shown in table 19.

Here Azotobacter is found to break down the nitrate and actually change it into the organic form with a resultant loss from solution.

Experiment 46. When the mannitol is replaced by glucose in a solution of the following composition the nitrogen balances vary only with regard to the quantity of nitrogen lost from solution.

Glucose	5.00 grams
K_2HPO_4	0.05 gram
$Ca(NO_3)_2 + 4H_2O$	0.316 gram
Deep well water	250.0 cc.

One hundred cubic centimeters of this solution pipetted into large Fernbach flasks received 0.5 gram $CaCO_3$. After inoculation of one with a very small quantity of a pure culture of *Azotobacter chroococcum,* the flasks were incubated for fifty-two days at 25°C. Table 20 summarizes the results obtained.

TABLE 20

Attack of nitrates by Azotobacter chroococcum

NUMBER OF CULTURE	TREATMENT	NITROGEN AS			SUGAR
		Organic and ammonia	Nitrous and nitric	Total	
		mgm.	*mgm.*	*mgm.*	*mgm.*
0	Check	0.88	17.14	18.02	1701.0
1	Inoculated	10.65	7.28	17.93	572.8
Differences		+9.77	−9.86	−0.09	−1128.2

Here again nearly the whole of the nitrate nitrogen reduced is to be found in the organic form. As the loss of nitrogen from solution is in this case negligible we may only draw attention to it now and reserve discussion to a later page.

To establish with certainty the fact of nitrate nitrogen attack and consumption another series of experiments may be cited which is typical of all the results obtained in this connection.

Experiment 47. A solution prepared according to Gerlach and Vogel constituted the basal medium.

Deep well water	500.00 cc.
K_2HPO_4	0.25 gram
NaCl	0.25 gram
$FeSO_4 + 7H_2O$	0.01 gram
Glucose	5.00 grams

To 250 cc. of this medium 0.316 gram Ca $(NO_3)_2 + 4H_2O$ were added. Fifty cubic centimeters of the nitrate solution and of the nitrate-free solution were placed in very large petri

TABLE 21

Nitrate attack by Azotobacter chroococcum

NUMBER OF CULTURE	TREATMENT	NITROGEN AS			GAIN OR LOSS OF NITROGEN
		Organic and ammonia	Nitrous and nitric	Total	
		mgm.	mgm.	mgm.	mgm.
	None				
A-0*	Control	0.76		0.76	
1	Inoculated	3.63		3.63	+2.87
2	Inoculated	4.43		4.43	+3.67
3	Inoculated	4.19		4.19	+3.43
4	Inoculated	4.50		4.50	+3.74
Average					+3.43
	Nitrate				
B-0*	Control	0.76	8.55	9.31	
1	Inoculated	8.87	0.36	9.23	−0.08
2	Inoculated	8.64	0.43	9.07	−0.24
3	Inoculated	8.71	0.20	8.91	−0.40
4	Inoculated	8.84	0.25	9.09	−0.22
Average					−0.24

* Calculated from experiments 46 and 49 made with the same solutions.

dishes which had been sterilized with 0.5 gram $CaCO_3$. Inoculation of some of the dishes with a pure culture of *Azotobacter chroococcum* was followed by incubation for twenty-four days at 25°C. The analytical results obtained are summarized in table 21.

The tabulated results require no further comment than has already been made. The nitric nitrogen is all, or nearly all, found in the form of organic or organized nitrogen.

The fact has thus been established that the nitrate nitrogen is organized by Azotobacter in its process of growth but no insight has been gained as to the *modus operandi* of this attack. A review of the reported data on nitrogen relations seems to point to the fact that the organism assimilates the nitrate in its early stages thus causing a loss of nitrogen from the solution, but later when the source of combined nitrogen is exhausted or nearly exhausted, a second physiological phase sets in, in which the cells assimilate atmospheric nitrogen, replacing thereby the losses which the solution underwent in its early stages. That this seems to be what actually takes place in the cultures of Azotobacter is evidenced by the accompanying experiments 51 and 55.

Experiment 51. Fifty cubic centimeters of a Vogel solution to which 0.126 per cent of $Ca(NO_3)_2 + 4H_2O$ had been added were used; after inoculation and incubation at 25°C. for varying lengths of time the cultures were analyzed with the results given in table 22.

Before discussing the results presented in the above table, a new series of experiments aiming at the same end will be related. The earliest incubation period of five days seemed to be too long to allow a close study of the early assimilation of the nitrate to be made. Shorter incubation periods were therefore observed to obtain the required data.

Experiment 55. Fifty cubic centimeters of the same solution as was used in the previous experiment were pipetted into sterile petri dishes of 20 cm. diameters, containing 0.5 gram of $CaCO_3$. After inoculation and incubation for varying periods of time the cultures were analyzed with the results set forth in table 23.

Although the actual amount of nitrogen unaccounted for is in many cases small, yet indications are that the *modus operandi* of the nitrate attack by *Azotobacter chroococcum* may be stated to be as follows; the organism utilizes the combined nitrogen (in the form of nitrates) as soon as placed in contact with it and

TABLE 22

Nitrate attack by Azotobacter

	DAYS OF INCUBATION	NITROGEN AS		SUGAR	NITROGEN UNACCOUNTED FOR
		Nitric and nitrous	Organic and ammonia		
		mgm.	mgm.	mgm.	mgm.
Control	5	8.66	0.47	476.8	
Inoculated	5	1.12	6.53	160.1	
Balance		−7.54	+6.06	−316.7	−1.48
Control	8	8.66	0.47	476.8	
Inoculated	8	0.88	7.41	0.0	
Balance		−7.78	+6.94	−476.8	−0.84
Control	13	8.66	0.47	476.8	
Inoculated	13	0.72	8.59	0.0	
Balance		−7.94	+8.12	−476.8	+0.18

TABLE 23

Nitrate attack by Azotobacter

	DAYS OF INCUBATION	NITROGEN AS		SUGAR	NITROGEN UNACCOUNTED FOR
		Nitric and nitrous	Organic and ammonia		
		mgm.	mgm.	mgm.	mgm.
Control	2	9.34	0.13	511.6	
Inoculated	2	5.79	3.70	361.0	
Balance		−3.55	+3.57	−150.6	+0.02
Control	5	9.34	0.13	511.6	
Inoculated	5	1.82	7.29	103.0	
Balance		−7.52	+7.16	−408.6	−0.36
Control	7	9.34	0.13	511.6	
Inoculated	7	1.02	8.39	12.4	
Balance		−8.32	+8.26	−509.6	−0.06

causes a loss of this element under special conditions that are not as yet completely understood. This loss may later be replenished by this same organism in the process of "later life." Apparently this second phase, phase of replenishment of the lost nitrogen, is directly connected with the second phase of the carbon metabolism studied in the preceding pages, since it appears to take place after the reducing sugar has either disappeared from solution or has been transformed into a non-reducing substance. That this assumption is justified is shown by the fact that the nitrates have a special importance in the carbon metabolism as is also shown by the data in the chapters on the carbon relations.

Nitrates and filtration of media

From the work of Allen (1919) we gather that filtration of a culture solution, under the conditions designed to remove the phosphates quantitatively, makes it unsuited for the development of *Azotobacter chroococcum*. Although the addition of calcium carbonate to the filtered solutions acted as a slightly beneficial agent in bringing about nitrogen fixation, it was not until phosphate was added in the form of tricalcium phosphate that any appreciable nitrogen fixation took place. Unfortunately the criterion used by Allen in drawing his conclusions[6] is subject to objection in the light of the above results so that in the following study the sugar consumption and production of bacterial substance was used as a measure of growth rather than the fixation of atmospheric nitrogen. The phosphates were replaced by nitrates, since some traces of phosphorus in the soluble form were undoubtedly present in the solution.

Experiment 69. A solution of the following composition was prepared and filtered, after a short standing at room temperature, over a coarse filter paper.

Deep well water	500.00 cc.
K_2HPO_4	0.25 gram
NaCl	0.25 gram
$FeSO_4 + 7H_2O$	0.02 gram
Glucose	5.00 grams

[6] Fixation of atmospheric nitrogen.

To 250 cc. of the filtered solution were added 0.316 gram $Ca(NO_3)_2 + 4H_2O$ and 50 cc. were pipetted into each of several 250 cc. Erlenmeyer flasks of Jena glass, containing 0.5000 gram of $CaCO_3$ weighed on an analytical balance. After sterilization and inoculation with a pure culture of *Azotobacter chroococcum* the flasks were incubated at 30°C. for varying periods of time.

Sugar determinations were made as well as determinations of the dry matter in the cultures after acidifying with HCl; the dry matter reckoned on the basis of the substance at 110°C. in vacuum over P_2O_5.

TABLE 24

DAYS OF INCUBATION	NO NITRATE			NITRATE		
	Cultural characters	Sugar consumed	Cell substance	Cultural characters	Sugar consumed	Cell substance
		mgm.	*mgm.*		*mgm.*	*mgm.*
3	No growth	0	0	Distinct turbidity	65.0	
5	No growth	0	0	Good growth	109.8	12
23	No growth	0	0	Good growth and pigment	411.5	62

Although the evidence seems at first sight to throw a shadow of doubt on the theory of phosphorus hunger in the filtered cultures, closer analysis shows it to corroborate the conclusions of Allen.

The nitrate used was tested for phosphorus by means of the ammonium molybdate reagent and found to be phosphorus free. The attempt was also made to avoid secondary reactions on any phosphates which might have passed through the paper by using $Ca(NO_3)_2$ instead of any other nitrate, but the aim might not have been fully reached. And this in spite of the work of Cameron and Hurst (1904) in which they found calcium nitrate to depress the concentration of the PO_4 ions in a solution of $Ca_3(PO_4)_2$ in presence of the solid phase.

III. DISCUSSION AND CONCLUSIONS

Azotobacter chroococcum Beij. when grown in synthetic solutions presents a complicated physiology. Its carbon relations seem to vary with the age of the culture, and are deeply affected

by the presence or absence of combined nitrogen in the solution. These carbon relations are in reality so closely connected with the nitrogen relations that to treat them separately would make the discussion abstract and unsound.

The fact that the cells seem to attack the sugar with a respiratory quotient of $CO_2:O_2 = \pm 1$ is apparently misleading and is not corroborated by a study of the sugar consumption. As we have seen we are forced to admit a first phase in the sugar metabolism, a phase that could well be named one of preparation, one in fact in which the sugar is worked up and changed into a compound or compounds of a non-reducing nature. From the study of the gas exchanges, it appears that the presence of nitrates aids in the better utilization of the sugar. (Tables 7 and 11.)

In this first stage, the "ferment power" of the organism is great and it is probably in this stage too, that the nitrates play an important rôle; in fact it is at this stage that the nitrate assimilation is at a maximum and evidence leads us to believe in a close relationship and interdependence of the two exalted functions, high "ferment power" and nitrate disappearance. Our filtration experiments before inoculation give us a proof of the paramount importance of nitrates in the process of sugar utilization, and, although the interpretation to be given to these facts is as yet unknown, evidence leads us to the belief that nitrates perform an intermediary function in the sugar fermentation and assimilation and it may well be that this preparation stage is directly dependent upon the formation of sugar-nitrate complexes analogous to the phosphate sugar complexes of Harden and Young.

In Allen's filtered solutions phosphates proved indispensable probably on account of their necessity in the formation of complexes of the hexose-phosphate type. The fact that nitrates proved to behave in a like manner leads us to the belief that Azotobacter cells may be capable of attacking complexes of the hexose-phosphate type as well as some homologues that involve the nitrate radicle.

The difference between the action of phosphates on zymase action and of the nitrates on cell metabolism lies in the fact that the nitrate proves to be actually organized by the cells whereas the phosphate in the work of Harden and Young is merely provisionally tied in an undisturbed form.

That this difference might be due to the fact that in the one case we are dealing with a "*figurated*" ferment while in the other we are only in presence of specific enzyme is not to be overlooked and investigation along this line may prove to us the possibility of this line of reasoning. In fact, the localization of the organized nitrogen in the Azotobacter cultures might be intracellular as well as extracellular and studies on this point would enhance our knowledge of the physiology of the organism concerned. Such studies are now under way in this laboratory.

In the second or maintenance phase, such complexes appear to be reworked, partially burned, partially utilized in the building of cellular substance and partially secreted in the surrounding medium in the form of soluble by-products. During this phase the nitrogen is actually assimilated, directly contrary to the belief of Hills.

A loss of nitrogen appears to take place during the first phase, a loss which, if slight, may be again made up in the second phase of development. The complication brought about by this first phase in the interpretation of the results does not render the term "ferment power" valueless or render useless the meaning adopted by Duclaux for this term but is only an example of what should be expected when studying the physiology of bacteria. Examples of a similar nature are not wanting in other branches of science and mention need only be made here of the limited value of the respiratory quotient in the study of the physiology of the Crassulaceae among plants and of Ascaris and Lumbricus among animals.

In concluding a word may be said of the practical interpretations of the above findings.

The activity of Azotobacter as a fixer of atmospheric nitrogen in the field is not easily demonstrated. Actual gains due to this organism in the nitrogen contents of a soil in the field are seldom

positively shown whereas it would be assumed that a nitrogen fixation from the atmosphere by the action of non-symbiotic nitrogen fixers should take place at an active rate to judge from laboratory experiments made in selective media and in absence of combined nitrogen. Yet a study of the subject will show that soils are only exceptionally free of nitrates and that these are easily washed away. It is therefore the belief of the present author that Azotobacter rather than serving as an active nitrogen (free) gatherer, may act to immobilize the nitrate nitrogen, taking the upper hand over the denitrifiers, and, to a considerable extent, stopping the mentioned percolation.

By this it should not be understood that the organism is hereby assumed to be lacking in all power of nitrogen fixation, but only that this function is not to be considered as an all-important phenomenon always active to the full benefit of man and to the detriment of the active organism itself, as it appears that "*all*" organisms choose the line of least resistance for obtaining and assimilating their food; and microörganisms are not an exception to the rule in spite of the arbitrary classification that is made of them into "*beneficial*" and "*non-beneficial.*"

That these experiments were made in solution does not detract from the conclusions derived therefrom, since we have seen that an obligate aerobic function such as nitrite formation, when studied by the methods used in this memoir may be advantageously compared with this function in soils.

IV. METHODS

A word is probably necessary on the methods used in the analysis of the cultures. The procedure used for the determination of ammonia, nitric and organic nitrogen on the same sample has been outlined by Davisson elsewhere (1918). The ammonia determinations were done by aeration over 5 grams sodium carbonate and subsequent distillation into standard acid. Subsequent treatment of the material in the aeration flask with 2.5 cc. of concentrated sulphuric acid, to destroy the carbonate, followed by 2 cc. of 50 per cent sodium hydroxid

and distillation into diluted acid (30 cc. H_2SO_4 in 30 cc. H_2O) for thirty minutes served to collect any ammonia resulting from the hydrolysis of the organic substances in the alkaline liquid. The solution was then diluted back to approximately 250 cc. and the nitric nitrogen therein contained determined by reduction and distillation from the alkaline solution in presence of 2 grams of Devarda's alloy. The acid solution containing the ammonia resulting from the hydrolysis of the organic matter was then transferred to the Kjeldahl flask containing the residue from the nitrate determination and the solution digested for the determination of organic nitrogen. The solution was digested until excessive frothing had ceased, then 10 grams potassium sulphate were added and digestion continued for one hour after the solution had become bluish-green. It was then distilled through an all-glass apparatus.

When it was necessary to determine the total nitrogen including the nitric nitrogen, the procedure developed in this laboratory was adopted (1919).

The sugar was determined in the solutions by clearing with colloidal iron and the centrifuge, using the clear liquid for the reduction of the Fehling solution as recommended by Shaffer (1914) and titrating the cuprous oxid by means of 0.05 N. potassium permanganate after dissolving it in Bertrand's solution. This solution was previously made pink by the use of the permanganate solution to avoid errors in the determination.

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REFERENCES

ALLEN 1915 Jour. Ind. Eng. Chem., **7**, 521.
ALLEN 1919 Ann. Missouri Bot. Gardens, **6**, 1–44.
ALLEN 1920 Ann. Missouri Bot. Gardens, **7**, 75–79.
BEIJERINK AND VANDELDEN 1902 Centr. f. Bakt. II., **9**, 3–43.
BONAZZI 1915 Jour. Agr. Res., **4**, 225–239.
BONAZZI 1919 Jour. Bact., **4**, 43–60.
CAMERON AND HURST 1904 Jour. Am. Chem. Soc., **26**.

DAVISSON 1918 Jour. Ind. Eng. Chem., **10,** 600.
DAVISSON AND PARSONS 1919 Jour. Ind. Eng. Chem., **11,** 306.
DUCLAUX 1898–1900 Traité de microbiologie, **1** and **3.**
GREAVES 1918 Soil Sci., **6,** 163–217.
HEINZE 1906 Landw. Jahr., **36,** 888–910.
HILLS 1918 Jour. Agr. Res., **12,** 183–230.
KOCH U. SEYDEL 1912 Centr. f. Bakt. II., **31,** 570–577.
KRAINSKY 1908 Centr. f. Bakt. II., **20,** 725–736.
KRAINSKY 1910 Centr. f. Bakt. II., **26,** 231–235.
KRZMIENIEWSKY 1908 Bull. Inter. Ac. Scie. Cracovie, Juillet, p. 929.
LIPMAN 1903 N. J. Exp. Stat. Rept., **24,** 217–285.
MAZÉ 1902 Ann. Inst. Pasteur, **16,** 195–346–433.
OMELIANSKY U. SEWEROWA 1911 Centr. f. Bakt. II., **29,** 643–650.
OMELIANSKY U. SIEBER 1913 Höppe-Zeyler Zeitschr. Physiol. Chem., **88,** 445–459.
SHAFFER 1914 Jour. Biol. Chem., **19,** 285.
STOKLASA 1908 Centr. f. Bakt., II., **21,** 484–509; 620–632.
STOKLASA 1908 Centr. f. Bakt., II., **21,** 620–632.
STRANAK 1909 Zeitschr. f. Zuckerind. Bohmen. Jahr. **33,** 599.